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ANIMAL PATHOLOGY.

[Reprinted from the PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE, December 18, 1918, VOL. xvi, No. 3, pp. 40–41.]

EXPERIMENTAL PNEUMONIA PRODUCED BY STREPTOCOCCUS HEMOLYTICUS.

BY MARTHA WOLLSTEIN AND S. J. MELTZER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Pulmonary lesions were produced by the insufflation of a strain of *Streptococcus hemolyticus* isolated in Texas and kindly given us by Dr. Avery. The cultures proved to be highly virulent for white mice. A quantity of the culture was insufflated intrabronchially into twenty-four dogs. The dose varied from 1 to 3 c.c. per kilo. Of these animals nine died in less than twenty-four hours, one died on the third day, and the rest were killed three to fifteen days after the inoculation. Dogs which survived more than two days went on to recovery, although they were ill for several days. Streptococcemia was found in all the animals which died early. It was also found in one of two dogs which was killed on the third day and in one of two killed on the fourth day after injection. Later than the fourth day no streptococci were found in the blood. Blood stained pus was present in the pleural cavity in three dogs, all having streptococci in the heart's blood. One of these animals died on the third day, one was killed on the third day and one on the fourth day. In the rest the pleura was normal. Empyema seemed to have developed before the third day.

The pneumonic lesion, in its early stage (twenty-four hours after injection), consisted of intense congestion, edema and small hemorrhages without pleurisy. After forty-eight hours the congestion and edema were still more marked and areas of bronchopneumonia had developed. The solid areas coalesced to some extent, but never became massive. The lungs in these dogs, even on the third day, were never very solid. The lesions involved usually more than one lobe. Resolution had begun on the fourth day, but in one instance there was a distinctly solid area of bronchopneumonia present on the seventh day. In the second week only areas of darker color were left. In no case had organization occurred.

Cultures from the lungs gave streptococci on the first, second, third and fourth days, but remained sterile on the fifth and sixth days. One case which presented an area of bronchopneumonia still unresolved on the seventh day gave a growth of streptococci from that area. Later than the seventh day the lungs contained no streptococci.

Microscopic examination of the sections made from lungs within twenty-four hours after insufflation of the culture showed congestion of all the vessels with the formation of thrombi in some of them. The alveolar contents consisted of red cells and coagulated serum, but there were practically no hemorrhages. On the second day the microscope showed that the alveoli were packed with polynuclear cells, little fibrin and many red blood cells. The solid areas surrounded inflamed bronchi. Infiltration of the framework of the lungs was present but not intense in any case. An abscess had formed in one lobe in one of the three cases with empyema.

The pulmonary lesion produced by the insufflation of the *Streptococcus hemolyticus* resembled the lesion found in human lungs from which the same organism was cultivated in that it was a bronchopneumonia with marked edema and a large amount of hemorrhage; it differed however from the human lesion by the lack of any tendency toward organization. In the experimental series empyema occurred in 12 per cent. of the cases and a pulmonary abscess was present only once.

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METHOD FOR THE ISOLATION AND RAPID IDENTIFICATION OF DYSENTERIC BACILLI.

By I. J. KLIGLER, Ph.D., and PETER K. OLITSKY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Bacillary dysentery is endemic in the United States, and localized epidemics occur from time to time. Our Army and Navy particularly stand in danger from this disease. Of late, confusion has arisen regarding its etiology, as a result of the failure to isolate *B. dysenteriæ* from the stools of patients. This led us to devise an improved method of isolation and rapid identification of these organisms.

The failure on the part of some workers to isolate the true dysentery bacilli from cases of clinical dysentery is attributable largely to two factors: (1) the improper selection of stool specimens for cultures, and (2) the use of unfavorable culture mediums.

1. Selection of Stool Specimens for Plating.—Too much emphasis cannot be laid on the importance of choosing a satisfactory sample of stool. If possible, one containing blood and mucus with little or no fecal matter should be used. It is essential to plate the stool directly or very shortly after it is collected. Experiments with artificial mixtures of the Shiga bacillus with feces show a 50 per cent. reduction in four hours and from 85 to 90 per cent. in twenty-four hours, when kept at room temperature.

2. Mediums Used for Plate Cultures.—Because of the extreme toxicity of dyes for dysentery bacilli, the selective inhibitive mediums used in the isolation of typhoid and paratyphoid bacilli are not suitable. Consequently the medium most widely used in this country is Endo's, and in England, MacConkey's. The shortcoming of the Endo plate as originally prepared has been realized by workers in this field, and various modifications have been suggested. Kendall and Day (1), Robinson and Rettger (2), and Kligler and Defandorf (3) successively proposed modifications. The first and last named authors recognized the fact that dysentery bacilli, particularly the Shiga variety, are highly sensitive to alkaline reactions and dyes,

and are either partially or wholly inhibited on the original Endo medium, which is distinctly alkaline and contains an excessive amount of the fuchsin indicator.

The Holt-Harris and Teague (4) eosin-methylene blue medium with meat infusion as a base proved entirely unsatisfactory because of its inhibitive effect on Shiga dysentery bacilli. Recently, however, Meyer and Stickel (5) have published comparative data indicating that the eosin-blue medium with veal agar or pepsin digest agar as a base gives better results than the Endo as modified by Kendall. The eosin-blue plate cultures show striking colony differentiation and have the added advantage of being at or near the neutral point.

Comparative tests were made with various mediums, the results of which are shown in the accompanying table. All strains of the Shiga bacillus used were isolated last summer and are undoubtedly more sensitive indicators of the favorableness of a given medium than old stock strains. The results indicate clearly that the veal infusion eosin-blue and modified Endo mediums are superior to the phosphate-peptone, the pepsin digest and the old unmodified Endo mediums. This superiority is also demonstrated when one compares the sizes of the colonies on the different plates.

Practically, the slight inhibitory effect of the veal infusion eosin-blue medium on Shiga bacilli is overcome by inoculating this type of medium as well as the modified Endo medium[1] with the same specimen.

PROCEDURE.

As a result of these tests, and experience in practical and class work, we recommend the following procedure in the isolation and rapid identification of dysentery bacilli from stools:

[1] The modified Endo's medium is prepared as follows: To veal or beef infusion 1.5 per cent. agar, titrated, and this is important, to p_H 7.6 to 7.8, is added 1 per cent. lactose and 1 per cent. by volume of decolorized basic fuchsin indicator. The latter is prepared by adding 1 c.c. of 10 per cent. basic fuchsin to 10 c.c. of 10 per cent. sodium bisulphite solution. Of course, the lactose and the indicator are added separately before plates are poured.

The eosin-blue medium is prepared by adding to the veal infusion agar, titrated to p_H 7.2 to 7.4, 1 per cent. lactose and 2 c.c. of a 2 per cent. solution of yellow eosin and 2 c.c. of 0.5 per cent. solution of water-soluble methylene blue.

*Comparison of Ratio of B. coli to B. dysenteriæ Colonies on Different Plating Mediums Inoculated from the Same Mixtures.**

Medium Used	Flexner Strains						Shiga Strains				Average Size of Colonies in Mm.
	23	24	26	28	31	J	100	109	2X	V. T.	
Veal infusion eosin-methylene blue	3:1	2:1	2.5:1	5:1	Spread	3:1	3:1	2:1	50:0	Crowded	1.5
Veal infusion Endo (Kligler's modification)	3:1	1:1	3:1	1.5:4	Spread	5:1	3:1	Spread	50:1	Crowded	1.75
Veal infusion Endo (old method)	4:1	48:0	7:1	5:1	48:0	35:0	200:1	40:1	200:0	2:1	0.5
Phosphate, eosin-methylene blue (Levine)	2:1	2:1	5:1	3.5:1	2:1	6:1	50:1	4:1	17:1	1:2	1.0
Peptic digest, eosin-methylene blue (Meyer and Stickel)	2:1	1:1	4:1	6:1	2.5:1	5:1	5:1	5:1	30:1	1:5	1.0
Peptic digest, Endo (Kligler's modification)	4:1	1:1.5	7:1	Spread	4:1	3.5:1	2.5:1	2.5:1	36:1	1:2.5	0.8
Peptic digest, Endo (old method)	Spread	1.5:1	7:1	Spread	4:1	.....	3:1	6:1	33:1	Spread	1.0

* The figures indicate the ratio of the number of colonies of *B. coli* to those of *B. dysenteriæ*. The marked inhibitive effect of the Endo medium prepared in the old way, titrating to +0.2 to phenolphthalein, on the Shiga types and the advantage of veal infusion medium are strikingly brought out in this table. "Spread" indicates that count was impossible as a result of confluence of colonies.

A fresh specimen of stool, preferably with blood and mucus, is collected and promptly cultured. A shred of bloody mucus, if present, is selected, washed three or four times with sterile saline, to remove all fecal matter, and spread successively on a veal infusion eosin-methylene blue and a modified Endo plate. The plates are then incubated from eighteen to twenty-four hours at 37°C.

The plates are now examined and the suspicious, colorless colonies inoculated into each of two differential tubes:

(*a*) A small tube containing 1 c.c. of a 0.5 per cent. dextrose broth.

(*b*) A double sugar tube on the principle of the Russell double sugar medium containing 0.1 per cent. dextrose, 0.5 per cent. mannite, and 1 per cent. Andrade indicator.

The colony is picked off with a small loop and inoculated first into the broth and then stabbed in and streaked on the double sugar tube.

After from two to four hours the broth tubes are usually sufficiently turbid for an agglutination test with a polyvalent antidysenteric serum. A faint visible turbidity is sufficient, and when it appears, 0.1 c.c. of a 1:50 dilution of a potent polyvalent antidysenteric serum is added and the tubes are reincubated for one hour. A definite clumping is a good presumptive test for the presence of dysentery bacilli.

The double sugar tube is incubated over night. A red butt without gas and colorless slant indicates a Shiga bacillus. If the entire tube is red and gas absent, it corresponds to a Flexner type. The surface growth is now washed off with saline and a confirmatory agglutination test is made with specific type and polyvalent serum. If desired, an agar slant and various sugar mediums may be inoculated for further study, or animal inoculation made.

By the use of the two tubes, one can obtain a presumptive diagnosis about a day after the collection of the stool—a matter of great importance for epidemiologic and therapeutic purposes.

CONCLUSIONS.

1. Fresh specimens preferably containing blood and mucus should be cultivated promptly.

2. Mediums for the isolation of dysenteric bacilli should not have a marked alkaline reaction or an excessive amount of inhibitive dyes. Mediums neutral or only slightly alkaline are most suitable. Veal infusion agar is a much more satisfactory base for differential plate cultures for the isolation of dysentery bacilli than is beef infusion or peptic digest agar. Eosin-methylene blue and modified Endo plates (pH 7.6 to 7.8) made with veal agar exert little or no inhibitive effects and permit the colonies to grow to almost twice their size on other mediums. Since the dye medium is apt to be somewhat inhibitive

for the Shiga type of dysentery bacillus, it should always be used together with a modified Endo plate.

3. The method given permits a presumptive and confirmatory diagnosis of bacillary dysentery in a very short time.

BIBLIOGRAPHY.

1. Kendall, A. I., and Day, A. A., *J. Med. Res.*, 1911, xxv, 95.
2. Robinson, H. C., and Rettger, L. F., *J. Med. Res.*, 1916, xxxiv, 363.
3. Kligler, I. J., and Defandorf, J., *J. Bacteriol.*, 1918, iii, 437.
4. Holt-Harris, J. E., and Teague, O., *J. Infect. Dis.*, 1916, xviii, 596.
5. Meyer, K. F., and Stickel, J. E., *J. Infect. Dis.*, 1918, xxiii, 48.

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EFFECT OF DRY HEAT ON THE BLOOD COUNT IN ANIMALS.

III. STUDIES ON LYMPHOID ACTIVITY.

BY JAMES B. MURPHY, M.D., AND ERNEST STURM.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 1 TO 3.

(Received for publication, October 17, 1918.)

The study of the function of the lymphoid tissue has, in the past, been productive of few leads of importance, due undoubtedly to the lack of methods for approaching the subject. It has not been possible, owing to the wide distribution of the lymphoid tissue throughout the body, to employ the method so productive in the study of deficiency of function in the glands of internal secretion brought about by partial or complete removal. Extensive investigations on animals and man after removal of the spleen, the chief lymphoid depot, has added little to our understanding of the function of this type of tissue. The loss under these conditions is so rapidly compensated for by hyperplasia of the lymphoid tissues that a diminished function cannot be detected. By utilizing the well known fact that lymphoid tissue is extremely sensitive to x-rays, we were able to develop a method by which practically the entire lymphoid tissue of the body can be destroyed with a minimum destruction of other tissue and slight, if any, effect on the general health of the animal.[1] The method consisted of small, repeated doses of x-rays distributed over from 7 to 21 days, depending on the size and resistance of the animal. The experiments carried out on the delymphocytized animals led to the following results: (1) destruction of the mechanism of resistance against implants of foreign tissue;[1] (2) lowered resistance to inoculated cancer grafts;[2] (3) destruction of established immunity to cancer;[3]

[1] Murphy, Jas. B., *J. Am. Med. Assn.*, 1914, lxii, 1459.

[2] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

[3] Murphy, Jas. B., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 1. Mottram, J. C., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917–18, xc, 1.

(4) lowered resistance to tuberculosis in mice,[4] guinea pigs,[5] and monkeys;[6] (5) lowered resistance to poliomyelitis in monkeys.[7]

From the experimental side we have been able to get suggestive results after the stimulation or apparent increased activity of the lymphoid tissue. The chick embryo, normally with no resistance against transplants of heteroplastic tissue, can be rendered resistant by suitable transplants of adult lymphoid tissue.[8] A single small dose of x-rays, sufficient to stimulate somewhat the circulating lymphocytes, increased the resistance of mice to autotransplants of spontaneous cancers.[9] Mice with high lymphoid counts resulting from splenectomy or from the reaction engendered in a cancer-immune animal after inoculation with a cancer graft show a marked increase in resistance to bovine tuberculosis.[4,10] All the results given above suggest, rather than prove, that the lymphocyte is an active agent in these varied processes. It seems necessary to test more completely the effect of stimulation by other means.

Many methods have been tested to produce an extensive and enduring increase in the lymphocyte and lymphoid tissue. Among these were the use of a number of drugs and dyes, ultra-violet light, and sunlight.[11] Except in the cases of the two latter agents, the results were unsatisfactory. Either the increase was too slight to be of experimental value, or not of long enough duration, or the incidental disturbance to the general health of the animal was too great. One highly promising method has been found; namely, the use of intense dry heat.

Method.

The source of the heat in these experiments was an electric bulb of frosted glass, giving the maximum heat for the amount of light generated. The method of application varied with the size of the

[4] Murphy, Jas. B., and Ellis, A. W. M., *J. Exp. Med.*, 1914, xx, 397.

[5] Morton, J. J., *J. Exp. Med.*, 1916, xxiv, 419.

[6] Taylor, H. D., unpublished observation.

[7] Amoss, H. L., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 115.

[8] Murphy, Jas. B., *J. Exp. Med.*, 1914, xix, 513.

[9] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.

[10] Taylor, H. D., and Murphy, Jas. B., *J. Exp. Med.*, 1917, xxv, 609.

[11] Taylor, H. D., *J. Exp. Med.*, 1919, xxix, 41.

animal and its ability to stand the higher temperatures. For mice an ordinary iron tripod 9 inches high and 5½ inches at the base was covered on the sides with cardboard. A space was left at the bottom for free circulation of air. This was placed over an electric "Sunbeam" heating lamp of 110 volts, 80 watts direct current. A small circular cage of the same size as the opening was placed on top of the tripod. This was made of galvanized iron with a wire mesh bottom (¼ inch mesh). The distance from the top of the lamp to the bottom of the cage was 3 inches. A thermometer was introduced parallel to and about ¼ inch below the bottom of the cage, directly over the light. After the light was turned on, the temperature was allowed to rise to 55°C. before the mouse was put into the cage. The animal was allowed to remain 5 minutes, this being about the time required for the temperature to rise from 55–65°C. If the temperature rose too rapidly it was found advisable to shut off the light until the thermometer registered between 57° and 59°C. and then allow it to increase again to 65°. For mice the temperature should never exceed 65°, as they can survive only 1 or 2 minutes at a higher temperature than this. A small piece of blotting paper was placed in the bottom of the cage just large enough for the mouse to rest on. In our experiments the loss was less than ½ of 1 per cent, and only occasionally were burns encountered.

For heating larger animals, such as rats or guinea pigs, a wooden box 9 by 6 inches and 4½ inches high was used with an electric carbon heating lamp 25 volts, 100 watts direct current, suspended directly over it. This lamp was surrounded by a metal reflecting cup. The distance from the lamp to the top of the box was 10 inches. The thermometer was placed at the level of the body of the animal. Rats and guinea pigs were found much more susceptible to higher temperatures than mice. A temperature of 60°C. or more caused extreme discomfort. The animals can, however, stand a more prolonged exposure at the lower temperature than can mice. For this reason it was found advisable to reduce the maximum temperature and increase the time of exposure for these animals.

Effect of Dry Heat on the Leucocytes in Mice.

Experiment 1.—Total and differential leucocyte counts were made on fifteen healthy mice of about the same age and size. 1 week later ten of these animals were heated over an electric lamp at a temperature ranging from 55–65°C. for 5 minutes. White cell and differential counts were made immediately after this procedure, and counts were also made on five remaining animals as controls. All the fifteen animals were counted again, 7, 14, and 21 days after heating. On charting the results of the counts it was found that immediately after heat exposure there was a uniform decrease in both principal types of white cells, the lymphocytes and the polymorphonuclear leucocytes, which averaged about 3,000 cells per c.mm. of blood. The count on the five unheated mice showed no appreciable change. 7 days after heating it was found that there was an increase of from 5,000 to 10,000 cells per c.mm., this increase being almost entirely in the lymphoid group. The polymorphonuclear leucocytes remained lower than they were in the original count. The blood picture in the five control mice was similar to that at the previous determinations. 14 days after heating, the lymphocytes had increased to a figure above the original count in some of the heated animals to the extent of from 12,000 to 14,000 cells per c.mm. of blood, while the polymorphonuclear leucocytes still remained below the original determination in practically all the animals. The control animals showed no change beyond that well within the bounds of experimental error. 21 days after heating, the lymphocytes, although several thousand cells above the original count, had fallen off somewhat from that of the previous week. The polymorphonuclear cells at this point showed a tendency to rise. These results are graphically recorded in Text-fig. 1 for the heated animals and Text-fig. 2 for the controls.[12]

Experiment 2.—White blood counts were made on ten healthy mice of approximately the same age and size. A week later five of these (Group I) were heated for 5 minutes at a temperature of from 55–65°C. Immediately afterward blood counts were made on these and the five unheated animals (Group II). A week later the control mice (Group II) were heated in the same manner as that employed with Group I the previous week. Counts were made on both groups at this period and repeated 7 and 21 days later. Group I showed an average decrease of 2,500 lymphocytes per c.mm. of blood after heating, while the polymorphonuclear leucocytes dropped more than 6,000 cells. Group II, which was used as the control up to this point, showed little change in the blood picture. Counts a week later on Group I, 1 week after heating, showed an average increase of over 6,000 lymphocytes per c.mm. above the normal level. The polymorphonuclear leucocytes had recovered somewhat from the effect of the heat, but were still below the normal. Group II at this time, after having been counted twice with

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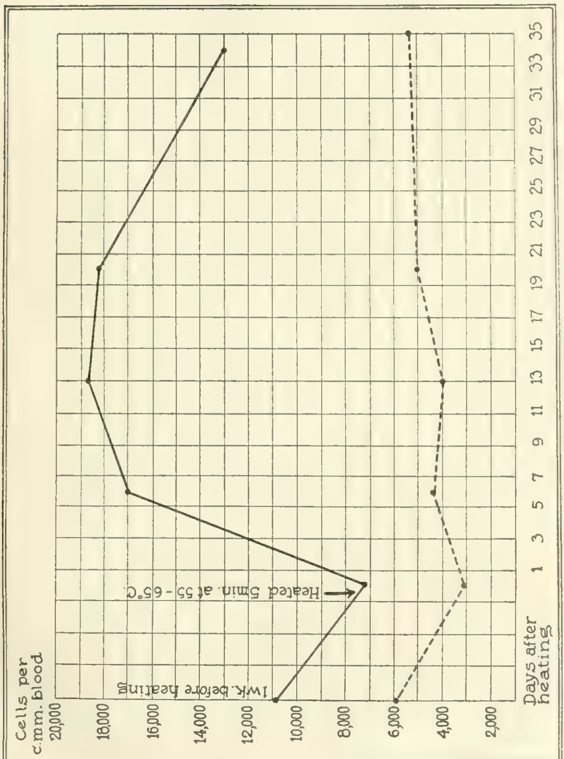
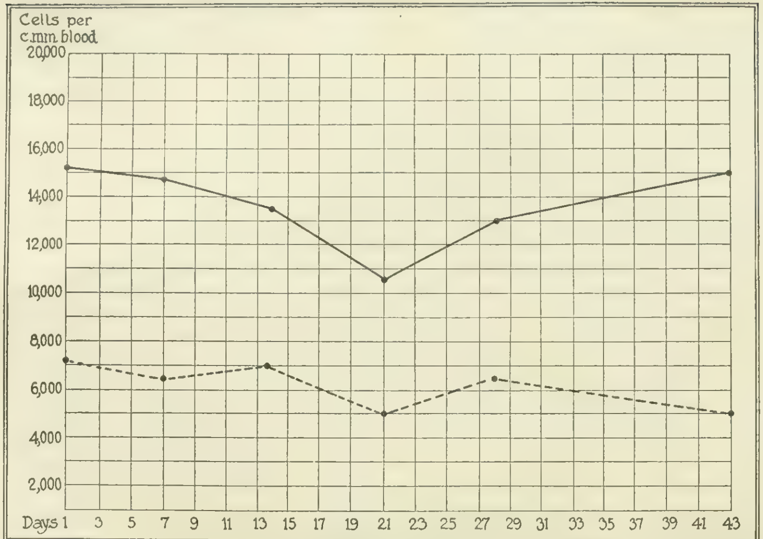
[12] From past experience it was found that if counts were made more often than once a week it caused too great a fluctuation in the count.

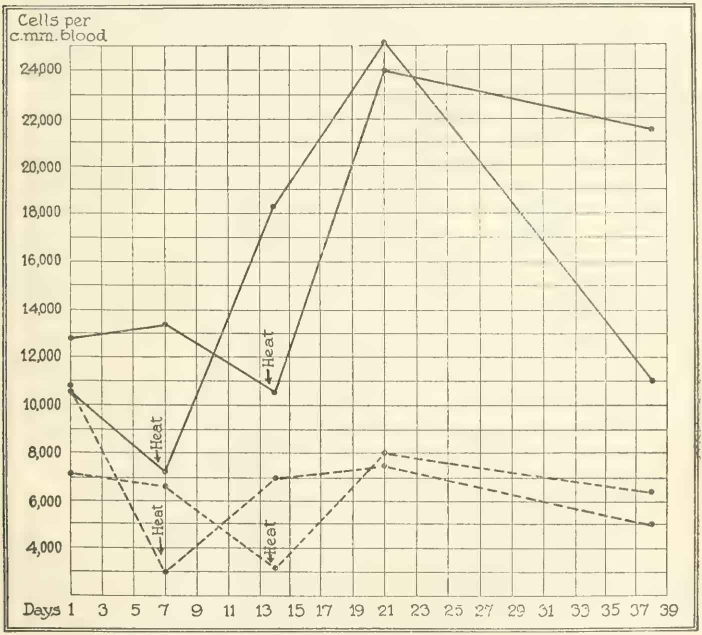


TEXT-FIG. 1. Composite curve of the white blood cell counts on ten mice before and after exposure to dry heat. ———Lymphocytes. ....Polymorphonuclear leucocytes.



TEXT-FIG. 2. Composite curve of the white blood cell counts on five normal mice of the same strain as those in Text-fig. 1. The animals were kept under the same conditions, and counts were made at the same time. ———Lymphocytes. ....Polymorphonuclear leucocytes.

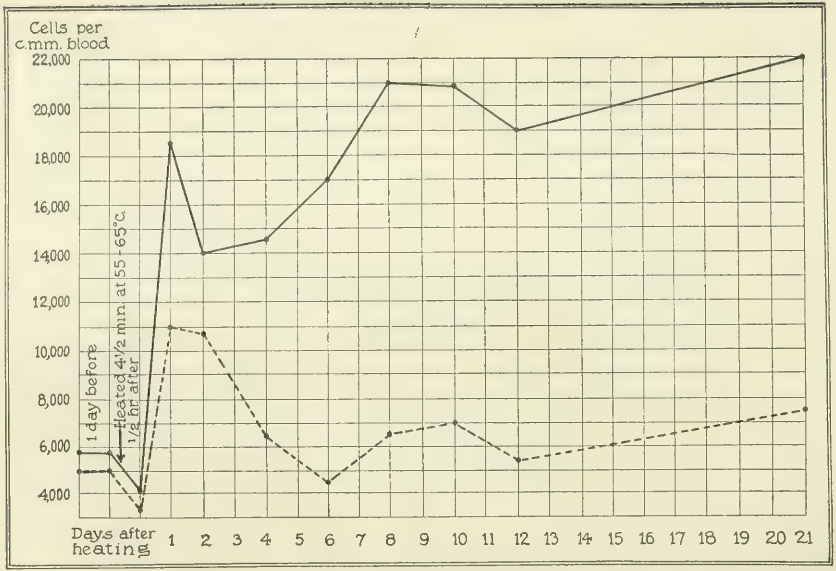
slight variation, showed the same characteristic drop in the blood count after heat was applied. After another 7 day interval the counts on Group I, 14 days after heating, showed an increase of 14,000 lymphocytes per c.mm. over the original count. Group II, 7 days after heating, showed an increase of 12,000 lymphocytes over the two previous counts. 14 days later Group I, 28 days



Text-Fig. 3. Composite curve of the white blood cell counts on two groups of mice, five in each group. One lot was heated a week after the first count, the others were heated 2 weeks after the first count with one intervening count. ———Lymphocytes. ....Polymorphonuclear leucocytes.

after heating, showed a count practically returned to its normal level, while Group II, 21 days after heating, was still several thousand cells above the normal count. This experiment is graphically illustrated in Text-fig. 3.

Experiment 3.—White blood counts were made on twenty healthy mice. 1 week later the entire twenty animals were heated for 5 minutes from 55–65°C.



TEXT-FIG. 4. Composite curve of the white blood cell counts on twenty healthy mice before and at intervals after heating. The first count represents the average for the whole lot. After heating they were counted in groups of five animals, the first group immediately after heating, the next 24 hours later, and then at intervals indicated on the chart. The last count represents the average of all twenty mice, ———Lymphocytes. ....Polymorphonuclear leucocytes.

and were then divided into four groups of five animals. Leucocyte counts were made on the first group immediately after heating, on the second 24 hours after heating, on the third 48 hours, and on the fourth 4 days after heating. Counts were made again on each group 6, 8, 10, and 12 days respectively after heating and again on all the animals 3 weeks after heating. The average of the counts for each day is shown graphically in Text-fig. 4, giving a continuous curve for the whole group.

Experiments 4, 5, and 6.—Three other experiments were carried out on mice in the same manner as those described above, with no variation in the result. These will not be given in detail.

In six experiments on mice it was found that after exposure of the animals to dry heat at 55–65°C. for 5 minutes there was a distinct fall in the total white blood cell count, both the polymorphonuclear leucocytes and the lymphocytes. The polymorphonuclear leucocytes recovered rather slowly, while the lymphocytes increased so rapidly that by the 2nd week after heating the count often reached a point 200 to 300 per cent above the normal figure.

The rectal temperature of mice after exposure to heat as described in the above experiments varies considerably, as shown by Table I.

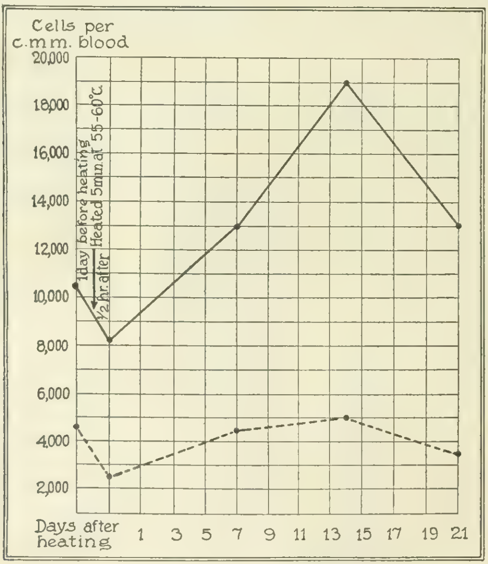
TABLE I.

Experiment No.	Before heat.	Directly after heat.	½ hr. after heat	1 hr. after heat.
	°C.	*°C.*	*°C.*	*°C.*
1	36.2	39.1	38.3	37.7
2	36.7	39.0	37.2	37.0
3	36.6	39.7	37.1	35.1
4	36.0	41.6	35.7	35.2
5	37.2	40.8	38.3	37.7

Morphology of Circulating Lymphocytes after Heating.

No attempt has been made in this study to differentiate the large and small lymphocyte, as it is practically impossible to establish a satisfactory dividing line between the two types. In general it may be said that the larger type predominates in the earlier stages of the stimulation, while later the smaller ones increase. The cells of both types are for the most part normal, healthy looking lymphocytes, and do not differ in appearance from those observed in the

normal animal. At a period of from 6 to 10 days after heating it was noted that a proportion of the lymphocytes were in the process of what appeared to be amitotic division. Every stage (Figs. 1 and 2) of this division could be observed. In some of the more extreme cases there were found from three to five cells in one microscopic field (Fig. 3).

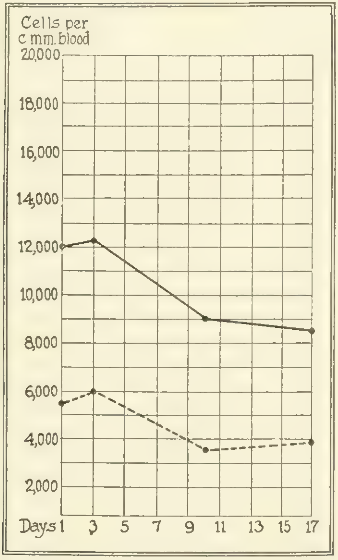


TEXT-FIG. 5. Composite curve of the white blood cell counts on five rats before and after heating. ———Lymphocytes. ....Polymorphonuclear leucocytes.

Effect of Heat on the Blood Picture in Rats and Guinea Pigs.

Experiment 7.—Blood counts were made on ten healthy rats of about the same age and size. The following day five of these animals were heated for 5 minutes at 55–60°C., counts being made immediately afterward both on these and on five control animals. The heated rats showed a decrease in both types

of cells similar to that observed in mice. The controls showed no marked change in their count. 7 days later counts showed the lymphocytes in the heated animals to be markedly increased, with the polymorphonuclear cells practically returned to their normal level. Control animals at this period showed a slight falling off in the count. 14 days after heating there was an average rise of over

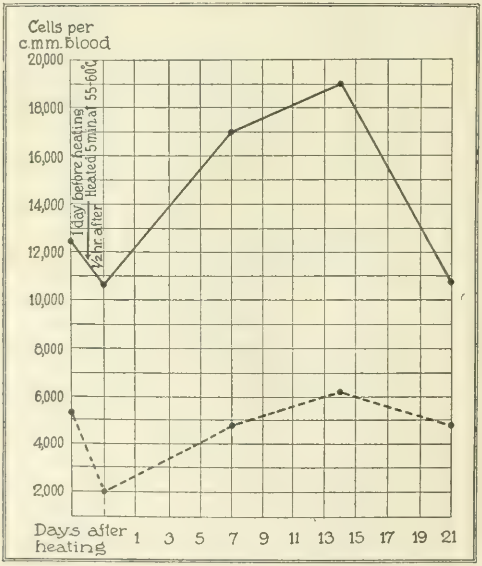


TEXT-FIG. 6. Composite curve of the white blood cell counts on five normal rats of the same lot as those in Text-fig. 5, kept under the same conditions, with counts made at the same time. ———— Lymphocytes. .... Polymorphonuclear leucocytes.

8,000 cells per c.mm. of blood, while the polymorphonuclear count remained about the same. 21 days after heating, the lymphocytes showed a tendency to decrease, but still remained well above the normal level. Text-fig. 5 is the composite curve of the heated animals and Text-fig. 6 that of the controls. Text-fig. 7 shows the curve of a typical rat after heating.

Several other experiments of a similar character on rats gave identical results, the changes being similar in every way to those observed in mice.

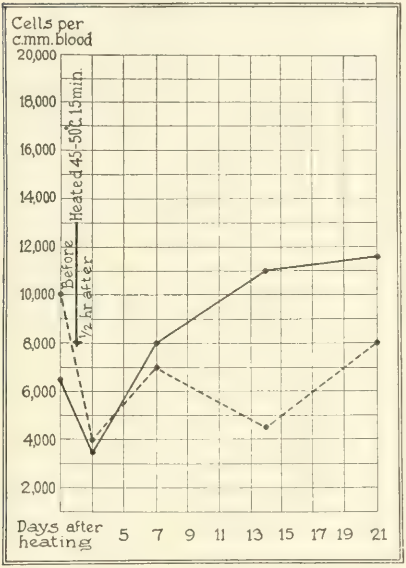
Experiment 8.—A total and differential leucocyte count was made on a fairly large guinea pig. The following day the animal was heated for 15 minutes at



TEXT-FIG. 7. Curve of the white blood cell counts of an individual rat after heating. ———Lymphocytes. ....Polymorphonuclear leucocytes.

45-50°C. and a count made shortly afterward. The lymphocytes showed only a slight decrease, while the polymorphonuclear leucocytes were reduced by 6,000 cells per c.mm. After this, counts were made at 7 day intervals for 3 weeks. They showed a steady increase in the lymphocytes, while the polymorphonuclears slowly regained their normal level and remained at that point (Text-fig. 8).

Several other experiments on guinea pigs gave identical results.



TEXT-FIG 8 The white blood cell counts of the guinea pig after exposure to heat. ———Lymphocytes. ....Polymorphonuclear leucocytes.

DISCUSSION.

Some years ago Wickline[13] made a study of the blood cells in American troops incidental to the complete physical examination made at intervals during residence in the Philippines. He showed that there was a marked increase in the relative and absolute number of mononuclear blood units, the increase being at the expense of the polymorphonuclear leucocytes. There was no marked change in the total white cell count. Chamberlain,[14] later, incidental to the study of the Arneth count in the tropics, confirmed and extended

[13] Wickline, W. A., *Mil. Surg.*, 1908, xxiii, 282.
[14] Chamberlain, W. P., *Mil. Surg.*, 1909, xxv, 48.

this interesting observation. There is not sufficient data, however, in the literature to determine either seasonal variation, the effect of altitude, or the various climatic conditions, to enable us to discuss our present results in relation to these observations in man. Neither are we prepared to discuss the underlying mechanism which brings about these remarkable changes in the blood picture from a single exposure to dry heat, nor will we attempt to offer an explanation for this at the present time. For purposes of further investigation it offers a method by which we can produce a marked and durable increase in the circulating lymphocytes, thus affording a further opportunity for the study of the function of these cells.

The second point of interest is the large number of lymphocytes found in the circulation in the process of what appears to be an amitotic division. The majority of biologists consider that amitotic division is a degenerative process, and they are inclined to cast doubt on the possibility of the development into normal functioning cells. In either case we know that after heating there is a large increase in what appear to be normal circulating lymphocytes of both the large and small type. At present it is impossible to say positively where they have arisen. It seems probable that there is a sufficient stimulation of the lymphoid centers to account for the increase.[15]

SUMMARY.

Animals subjected to dry heat for a short period show a sharp fall in the total white blood count, both the polymorphonuclear leucocytes and the lymphocytes taking part in the fall. Following this there is a slow recovery on the part of the polymorphonuclear leucocytes, which generally require several weeks to regain their normal number. The lymphocytes rise rapidly after the initial fall and continue to rise for 2 or 3 weeks. This increase often amounts to a gain of over 200 to 300 per cent above the normal count for the animal. The observations were made on mice, rats, and guinea pigs.

The circulating lymphocytes during the more active stage of stimulation after heating show numerous examples of amitotic division.

[15] Nakahara, W., *J. Exp. Med.*, 1919, xxix, 17.

EXPLANATION OF PLATES.

Plate 1.

Fig. 1. Microphotograph from a blood film of a mouse 6 days after an exposure to heat, showing various stages of amitosis in the lymphocytes.

Plate 2.

Fig. 2. Drawing of various stages of amitosis seen in the lymphocytes of the blood of a mouse 8 days after exposure to heat.

Plate 3.

Fig. 3. A single microscopic field from a blood film of a mouse 8 days after exposure to heat, showing four irregular lymphocytes.

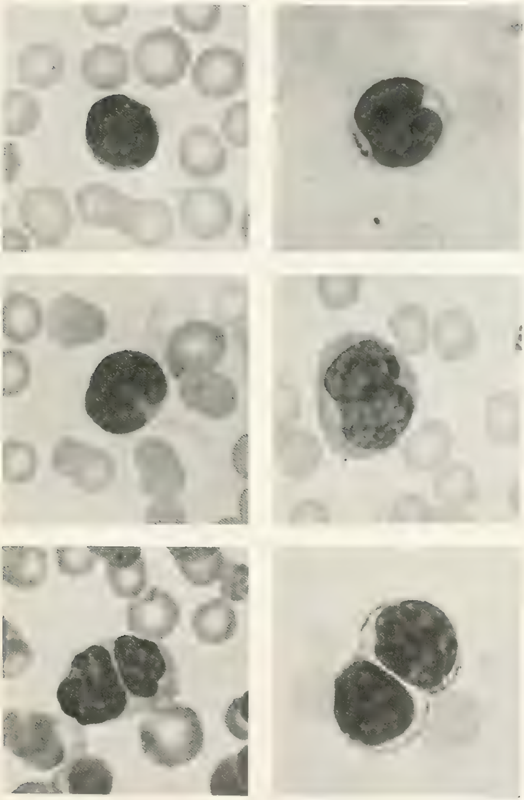


FIG. 1.

(Murphy and Sturm: Effect of dry heat on blood count. III.)

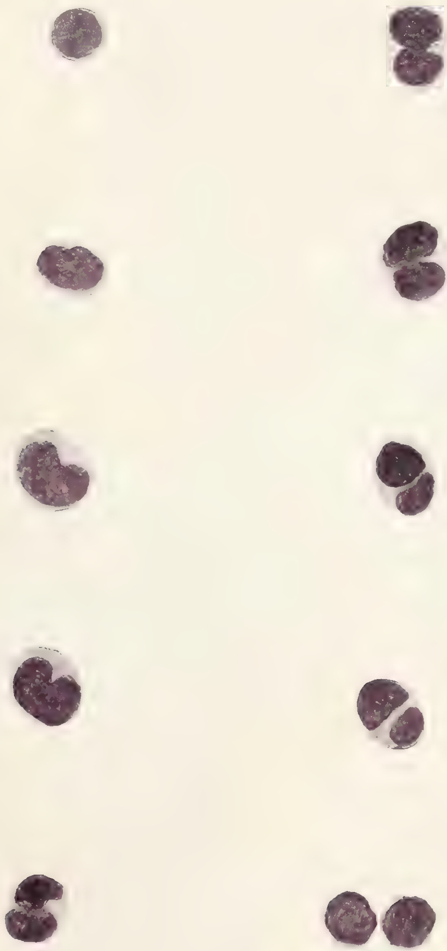


Fig. 2.

(Murphy and Sturm: Effect of dry heat on blood count. III.)

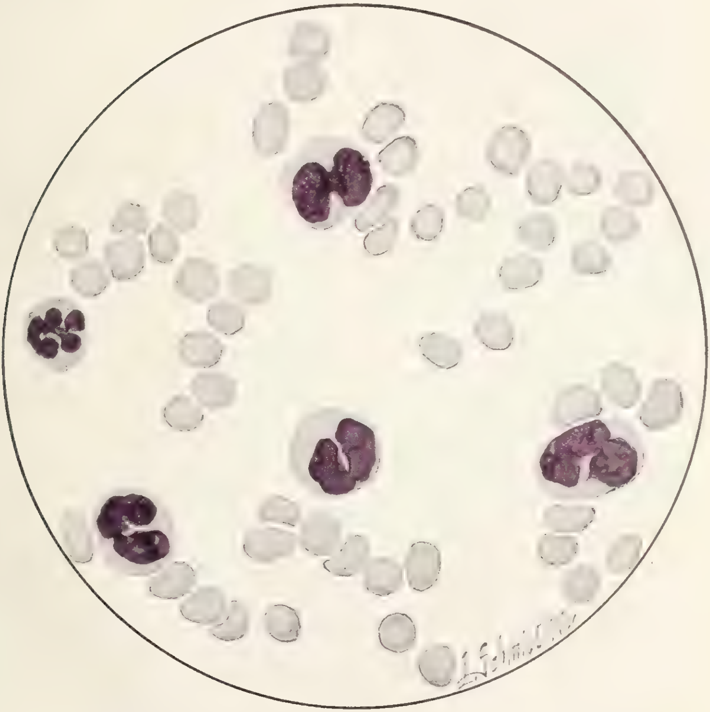


Fig. 3.

(Murphy and Sturm: Effect of dry heat on blood count. III.)

[Reprinted from The Journal of Experimental Medicine, January 1, 1919, Vol. xxix, No. 1, pp. 17–23.]

THE SOURCE OF THE LYMPHOCYTOSIS INDUCED BY MEANS OF HEAT.

By WARO NAKAHARA, Ph.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 4 to 7.

(Received for publication, October 17, 1918.)

A recent article from this laboratory reported experiments showing the effect of dry heat on the blood picture in mice.[1] After a 5 minute exposure of the animals to heat ranging from 55–65°C. there was an immediate fall in the circulating white cells. This was followed by a sharp rise in the lymphocytes which increased to a point about 200 per cent above their initial number. The increase extended over from 2 to 3 weeks and later gradually subsided to the normal level. The polymorphonuclear leucocytes participated in the initial fall but recovered very slowly and rarely went above the normal number. The lymphocytes resulting from this stimulation appeared to be normal, healthy cells of both the large and small type. It was noted that during the most active proliferative stage a number of lymphocytes were in the process of typical amitotic division. It seemed doubtful, however, in the light of the almost generally accepted opinion of biologists that this process ever gives rise to normally functioning cells. As the heat stimulation produces normal looking cells, it seemed desirable to study the other possible sources of origin.

Source and Type of Material.

The material for this study was collected by Murphy and Sturm[1] from a number of mice carried as a parallel to an experiment in which blood studies were made. These mice were of the same stock and about the same age. They were subjected to an exposure of dry heat for 5 minutes at a temperature ranging from 55–65° C. Several

[1] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 1.

of these mice were killed immediately after the heat exposure and the others in groups at intervals of 2, 4, 6, 8, 12, and 14 days. The spleen, lymph glands, and other organs were fixed in Flemming's, Bouin's, or Zenker's fluid. Sections were cut from 5 to 12 microns thick, and most of them stained with Heidenhain's iron-hematoxylin, eosin-methylene blue, and a few with Ehrlich's triacid stain. Of this material, the spleen and lymph glands have been studied and the results will be briefly described.

OBSERVATIONS.

Spleen.—The spleen becomes decidedly changed immediately after the treatment with heat. An examination reveals that there are a large number of cells degenerated or tending to degenerate (Fig. 1). Pyknosis is present not only in the cells of the nodules, but also in the pulp. The cell mass of the germinal center, however, seems always to remain unaffected (Fig. 2). There is no evidence of proliferative activity in the germinal center in the nodule, and only rarely can a mitotic figure be seen. There is also an apparent decrease in the number of the megalokaryocytes.

Sections taken 48 hours after the treatment present an appearance similar to that seen in the normal animals, the differences being an excessive number of necrotic cells, a relative scarcity of megalokaryocytes, and a great number of mitotic figures in the germinal center in the nodules. The active proliferation of cells in the germinal center (Fig. 3) is of significance in view of the fact that in the normal spleen of the adult the cells in the germinal center are not usually actively proliferating, while in infancy, when the lymphoid elements are actively formed, mitosis is of usual occurrence. These facts suggest that the occurrence of the mitotic figures in the germinal center, following the widespread degeneration, is primarily for the purpose of restoring the normal condition in the organ. The phenomenon may thus be regarded as one of overregeneration.

Sections taken 4 days after the treatment show that the spleen is well on the way to recovery (Fig. 4). Here necrotic cells are much less abundant, although they persist, especially in the pulp and in the periphery of the nodule. As in the normal spleen, the association

of mitotic figures with the germinal center is inconstant. The megalokaryocytes, which had been more or less decreased, are now present in fairly large numbers.

A condition similar to that last described is shown in all the sections taken later, after the treatment. That the megalokaryocytes are more, and necrotic cells less abundant in sections taken later may be looked upon as indicating complete recovery.

Macroscopically, the organ apparently undergoes marked enlargement at about the 6th to 8th day after the treatment. I have observed on the 8th day a spleen approximately four times the normal size. At the 14th day the size of the organ is seen to be normal. The size of the spleen varies greatly, even among apparently normal mice, but the fact of its enlargement after the treatment seems, in a general sense, to be indisputable.

Mesenteric Lymph Gland.—The cortex of the mesenteric lymph gland becomes full of necrotic cells immediately after the treatment (Fig. 5). The central cell mass of the nodules (germinal center) is apparently normal, but as a rule, no mitotic figures are seen in it. Numerous necrotic cells are observed in lymph cords, and no mitotic figures are seen. The pulp spaces are apparently normal.

After 48 hours the nodules are almost free of necrotic cells, although these cells are present in other parts of the cortex. Cells in the germinal center are actively proliferating, as evidenced by large numbers of mitotic figures in that region (Fig. 6). In lymph cords numerous necrotic cells and few mitotic figures are seen. The appearance of large multinucleated cells, probably phagocytic, is noted in the pulp spaces.

Conditions after the 4th up to the 14th day are apparently normal (Fig. 7). Mitosis in the germinal center is also of normal frequency, judging from the number encountered in sections.

Grossly, there is a general enlargement of the gland at about the 8th day after the treatment. The lymph glands taken at 14 days are approximately of normal size.

Inguinal Lymph Gland.—The changes observed here are exactly similar to those found in the mesenteric gland. Numbers of necrotic cells (Fig. 8) and suppression of the proliferative activity in the germinal centers are the fundamental characteristics immediately

after the treatment. After 48 hours necrotic cells become almost entirely eliminated from the cortex, and the germinal center assumes an appearance of great proliferative activity (Fig. 9). In the medulla there are still a number of necrotic cells persisting, but isolated cases of mitosis in lymph cords are not infrequent.

Histologically, the gland after the 4th day of the treatment is apparently normal. Here, also, as in the mesenteric gland, there is an apparent abnormal enlargement of the gland at about the 8th to 10th day after the treatment.

DISCUSSION.

One of the most striking findings in the observations described above is the complete parallel in the changes undergone by the splenic cells and the cells of the lymph glands. The first change observed in the spleen and in the two kinds of lymph glands, after the treatment with heat, is the widespread degeneration in their cell elements. Within 48 hours, or perhaps much earlier, the germinal centers become active, as demonstrated by the abundant mitotic figures in them, and they thus function in the restoration of the cellular elements of the organs. The germinal centers, however, later become inactive again.

In all the cases, both in the spleen and in the lymph glands, there is a marked increase of mitotic figures in the germinal center, following the general necrosis. The frequency of mitosis in the region apparently exceeds by far that in the normal animal, and it is natural to suppose that the enhanced cell proliferation accounts for the overproduction of lymphocytes. That the cell multiplication here is more than compensation for the degenerated cells is evident in light of the fact that dimensional enlargement of the organ, often well marked, follows the activity of the germinal center. The enlarged spleen and lymph glands are filled with normal cells, and it seems to be indisputable that excessive proliferation of the cells is responsible for their altered appearance. An attempt was made to ascertain whether there is definite increase in number and expansion in the area of germinal centers, after they are stimulated, but it has not been successful because of the unfavorable nature of the structure for such study.

It has been emphasized that a large number of cells in the spleen and in the lymph glands degenerate from the effect of heat immediately after its application. If, then, the cells in the spleen and lymph glands are a source of lymphocytes, there should be a fall in the number of these cells in the circulating blood following the application of heat. Murphy and Sturm have shown this to be the case. This corollary, taken together with other better known facts, tends to warrant the conclusion that the source of the induced lymphocytosis is at least partly the lymphoid cells in the spleen and lymph glands.

Results of recent experiments carried on in this laboratory point to the fact that a certain dose of x-rays can be used to produce a lymphocytosis similar to that brought about by heat.[2, 3] The results of blood counts after suitable treatment of the animal with this agent prove that the lymphocytic change is parallel to that in the case of heat treatment, with the characteristic fall in lymphocyte count preceding the marked rise.

Attention may be called to the results of Heineke[4] and Warthin,[5] who have shown that the effect of x-rays on lymphoid tissues is, in the main, similar to that of heat, as described in the present paper. Their data, however, do not throw any light upon the question as to whether there is any proliferation of lymphoid cells above the normal in the process of regeneration, as they did not make observations during the critical period after the x-ray treatment, when an excessive multiplication of the cells may possibly take place. For its satisfactory solution, therefore, the problem must be reinvestigated from a standpoint different from that of the earlier workers.

The induced lymphocytosis is not due to the direct action of heat on lymphoid cells, but is due to proliferative activity on the part of the cells. In other words, heat is primarily a destructive agent of the lymphoid cells, but it causes secondary multiplication of the cells, thus bringing about pronounced lymphocytosis.

[2] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.

[3] Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75.

[4] Heineke, H., *Mitteil. Grenzgeb. Med. u. Chir.*, 1905, xiv, 21.

[5] Warthin, A. S., *Physician and Surg.*, 1907, xxix, 1.

SUMMARY AND CONCLUSION.

A striking number of mitotic figures have been observed in the germinal center of the spleen and lymph glands during the regeneration of the cellular elements of these organs after the destructive effect of heat. This enhanced cell proliferation is interpreted as more than compensating for the degenerated cells, because of the subsequent enlargement of the organs. It has also been pointed out that the characteristic decrease in the number of lymphocytes immediately after the heat treatment is always accompanied by an extensive cell degeneration in spleen and lymph glands at the corresponding period.

On this basis it seems evident that the pronounced lymphocytosis induced by means of heat treatment of the animal is due, at least in part, to the enhanced proliferative activity of germinal centers in the spleen and lymph glands, reacting to the destructive effect of heat upon lymphoid cells.

EXPLANATION OF PLATES.

Plate 4.

Fig. 1. A part of the spleen, immediately after the treatment, showing necrotic cells around the inactive germinal center. × 450.

Fig. 2. The same, showing normal cells of the germinal center (above), and necrotic cells around it (below). × 1,000.

Plate 5.

Fig. 3. Germinal center of the spleen, 48 hours after the treatment. *M*, mitotic figures. × 1,000.

Fig 4. Germinal center of the spleen, 4 days after the treatment, showing the condition to be apparently normal. × 1,000.

Plate 6.

Fig. 5. Part of cortex of the mesenteric lymph gland, immediately after the treatment, showing marked necrosis. × 1,000.

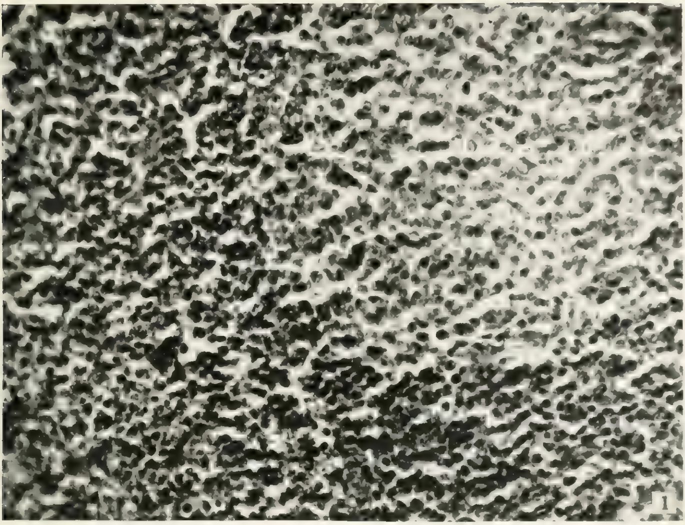
Fig. 6. Germinal center of the mesenteric lymph gland 48 hours after the treatment. *M*, mitotic figures. × 1,000.

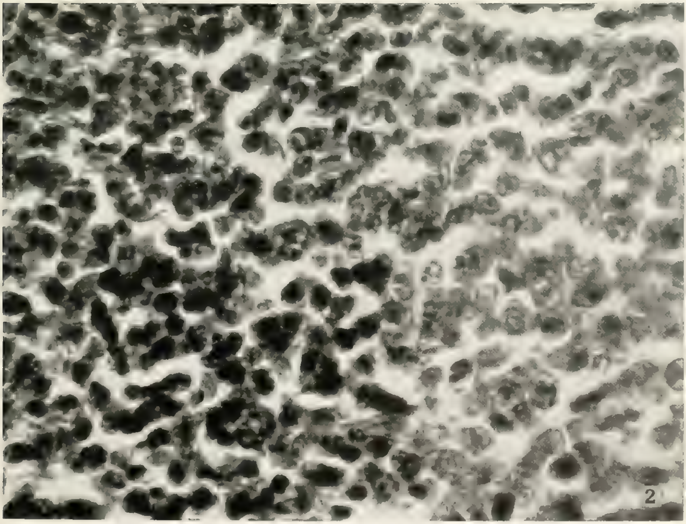
Fig. 7. The mesenteric lymph gland 12 days after the treatment, showing the germinal center (left) and cells around it. × 1,000.

PLATE 7.

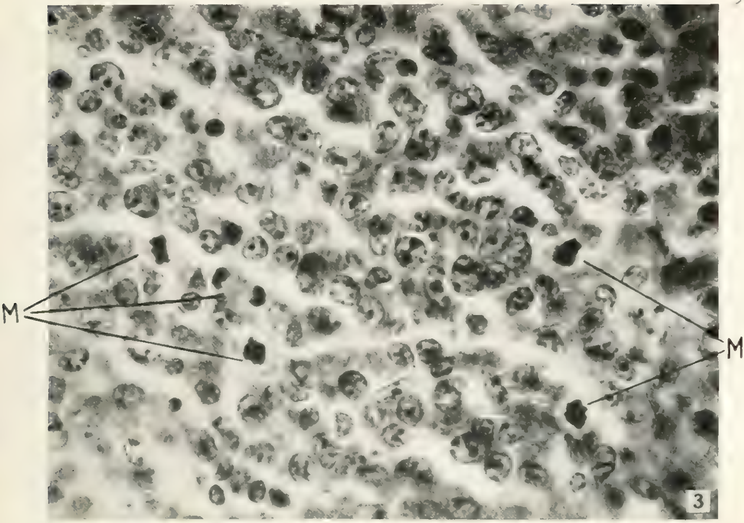
FIG. 8. Part of the medulla of the inguinal lymph gland, immediately after the treatment, showing necrotic cells. × 1,000.

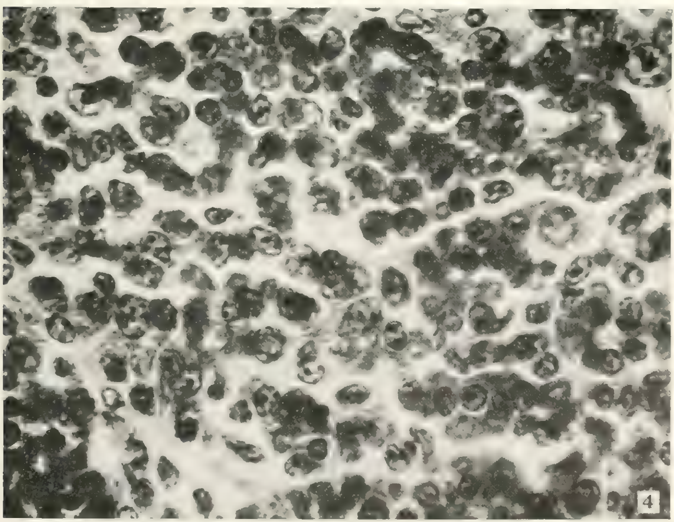
FIG. 9. Germinal center of the inguinal lymph gland 48 hours after the treatment. *M*, mitotic figures. × 1,000.



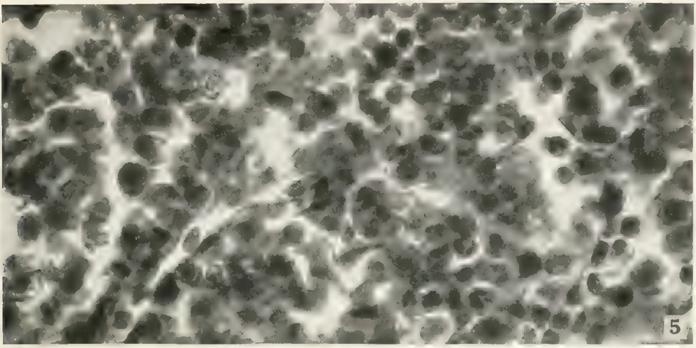


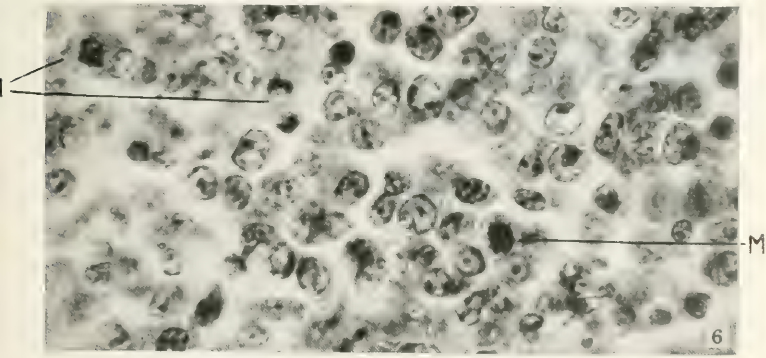
(Nakahara: Lymphocytosis induced by means of heat.)

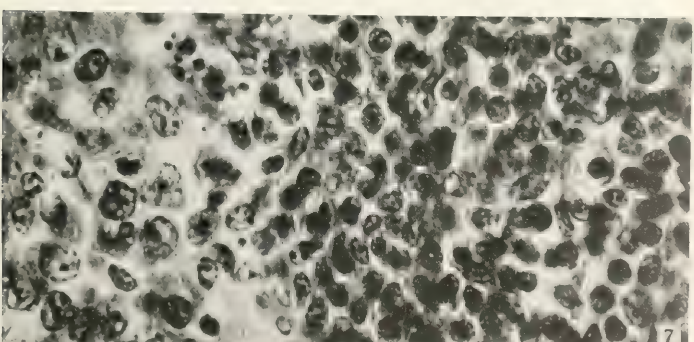




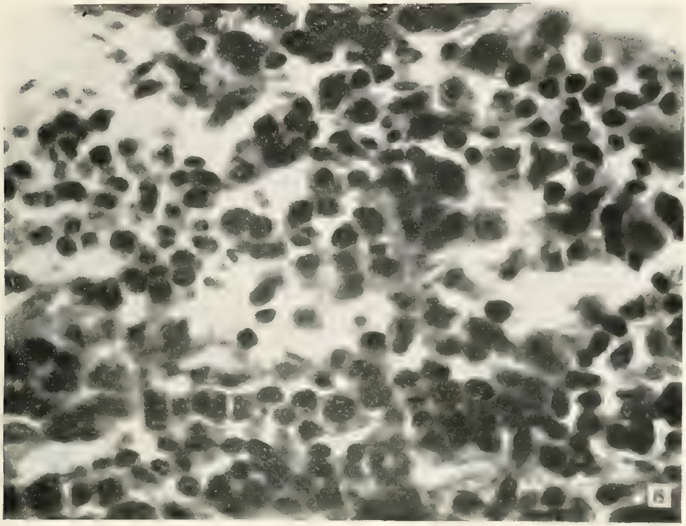
(Nakahata: Lymphocytosis induced by means of heat.)

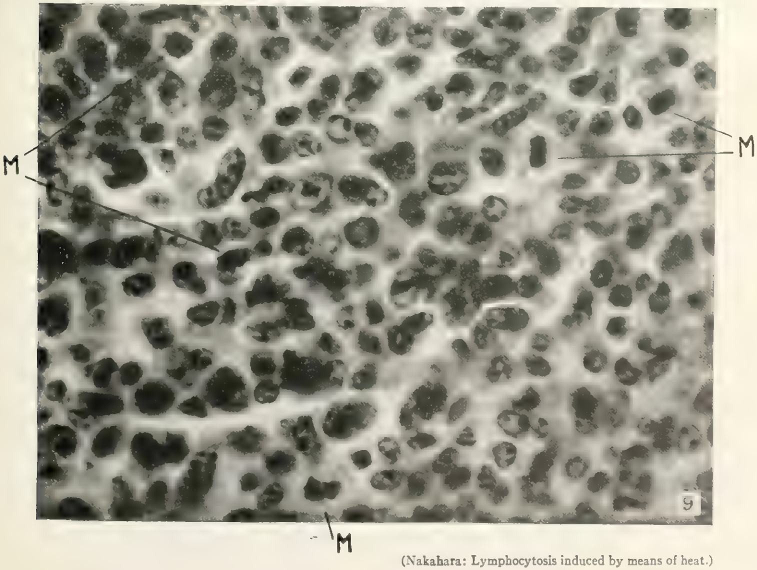






(Nakahara: Lymphocytosis induced by means of heat.





(Nakahara: Lymphocytosis induced by means of heat.)

[Reprinted from The Journal of Experimental Medicine, January 1, 1919, Vol. xxix, No. 1, pp. 25–30.]

THE LYMPHOCYTES IN NATURAL AND INDUCED RESISTANCE TO TRANSPLANTED CANCER.

IV. Effect of Dry Heat on Resistance to Transplanted Cancer in Mice.*

By JAMES B. MURPHY, M.D., and ERNEST STURM.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 18, 1918.)

The striking histological difference between a cancer graft in an immune animal and in a susceptible animal is the early appearance of large numbers of lymphocytes in the former and the relative absence of these cells in the latter. This fact has led some observers to venture the opinion that the lymphocytes may be a factor in the resistance to these growths. Da Fano[1] carried out extensive histological studies of this reaction about the cancer graft in immune animals and also extended his observations to changes in the cells of the subcutaneous tissue of the body. He demonstrated a marked increase in the lymphoid elements about the cancer graft and in the tissues, and likewise showed an increase in the closely related group of plasma cells. Later Murphy and Morton[2] showed that mice potentially immune to cancer developed a marked lymphocytosis after inoculation with a cancer graft. These investigators also showed that potentially immune animals can be rendered susceptible to cancer inoculation if the lymphoid tissue is depleted by means of x-rays. Murphy and Taylor[3] extended the latter observation and showed that immune animals of a tested resistance can be made again susceptible to inoculation after depletion of the lymphoid tissue.

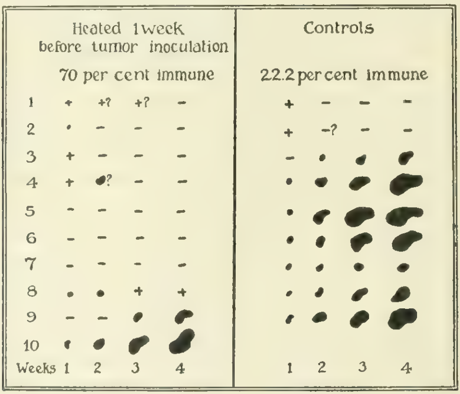
*This investigation was carried out by means of funds from the Rutherford Donation.

[1] Da Fano, C., *Z. Immunitätsforsch., Orig.*, 1910, v, 1.

[2] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

[3] Murphy, Jas. B., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 1.

It has been deemed advisable to test the effect of induced lymphocytosis in the animal on resistance to inoculated cancer. The amount of stimulation of the lymphocytes induced by splenectomy[4] and small doses of x-rays[5] did not prove sufficient to influence markedly the course of these highly resistant transplantable tumors. However, with the development of the more extensive and enduring stimulation induced by heat we have an opportunity of testing the effect of such a reaction on cancer resistance.



TEXT-FIG. 1. Rate of growth of the Bashford Adenocarcinoma No. 63 in mice heated 1 week before inoculation contrasted with the rate of growth in control animals.

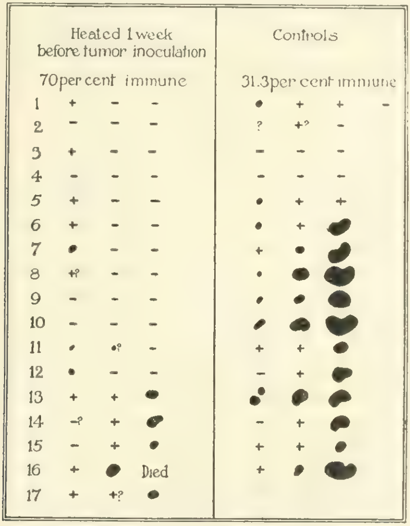
Experiment 1.—Ten mice of about the same age and weight were heated for 5 minutes at 55–65°C. over an electric heat lamp. 1 week later the animals, together with ten healthy controls, were inoculated subcutaneously in the groin with a strain of a 3 week old transplantable cancer (Bashford Adenocarcinoma No. 63). The rate of growth of the tumors was charted at weekly intervals thereafter. At the end of 4 weeks the heated animals showed only three tumors, with an immunity of 70 per cent. Of the nine control animals surviving, seven developed tumors, and only two were immune. This per cent of immunity (22.2)

[4] Murphy, Jas. B., and Ellis, A. W. M., *J. Exp. Med.*, 1914, xx, 397.

[5] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.

is in striking contrast to the 70 per cent in the heated animals. The results of this experiment are shown in Text-fig. 1.

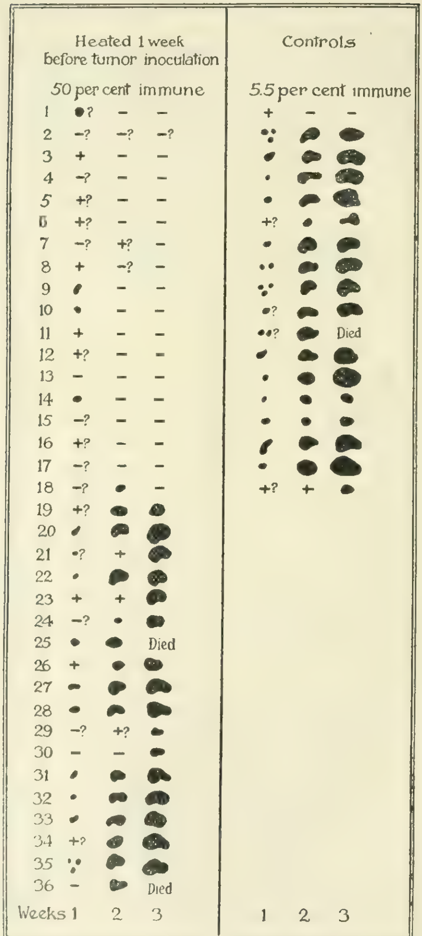
Experiment 2.—Seventeen mice were heated in the manner described in the previous paragraph. A week later these animals, with sixteen control mice, were inoculated with a strain of the Bashford Adenocarcinoma No. 63. Weekly measurements were made of the developing tumors. The heated animals showed five out of seventeen with tumors, or an immunity of 70 per cent. Eleven of the sixteen control animals developed tumors, the immunity being 31.3 per cent.



TEXT-FIG. 2. The same as Text-fig. 1.

The difference in immunity between the heated animals and the controls presents a striking contrast. The result is shown in Text-fig. 2.

Experiment 3.—Thirty-six healthy mice of about the same age and size and from the same stock were heated for 5 minutes over an electric heat lamp at a temperature ranging from 55–65°C. A week later these animals, with eighteen normal mice, were inoculated with a $2\frac{1}{2}$ week old Bashford Adenocarcinoma No. 63. After 3 weeks eighteen of the thirty-six heated animals had developed tumors, showing an immunity of 50 per cent, while seventeen of the eighteen con-



TEXT-FIG. 3. The same as Text-fig. 1.

trols had developed tumors, the immunity being only 5.5 per cent. This experiment is shown graphically in Text-fig. 3.

The three foregoing experiments, carried out on over 100 mice, show that animals whose lymphocytes have been stimulated by dry heat have a much higher resistance to transplanted cancer than control mice inoculated with the same tumor. The general health of animals subjected to this treatment did not seem in the least affected. There was no loss of weight, no roughening of the hair, or other indication of disturbance. This difference in resistance was manifest both when the tumor inoculated gave a relatively low per cent of takes, and when the tumor was highly virulent and gave a high per cent of takes.

DISCUSSION.

The absence of any acceptable demonstration of antibodies to explain cancer immunity suggests strongly that this type of resistance probably comes under the head of cellular immunity. The evidence connecting the lymphocyte with the resistance to transplantable cancer may be summed up briefly as follows: (*a*) the presence of lymphocytes and related cells about a cancer graft in immune animals, and the relative absence of these cells around such a graft in highly susceptible animals; (*b*) the general changes which take place in the cellular elements of the tissues of animals potentially immune to cancer (Da Fano); (*c*) the lymphocytic crisis in the circulating blood of potentially immune animals after inoculation with cancer; (*d*) destruction of potential cancer immunity by depletion of the lymphoid elements with x-rays; (*e*) destruction of established cancer immunity by the same means; (*f*) the marked increased resistance to cancer after artificial stimulation of the lymphocytes.

It would be difficult to adapt the foregoing facts so that they would fit either into the Ehrlich theory of cancer immunity or that of the Bashford school of stroma reaction. Neither of these explanations seems tenable in the light of the present results. That other factors than the lymphocytes are involved in the process of cancer immunity seems more than probable. It can at least be said with a degree of certainty that we have in the lymphoid elements an important link in the process of so called cancer immunity.

SUMMARY.

The marked and durable stimulation of the lymphoid elements induced by dry heat applied to the animal results in the establishment of a high degree of immunity to certain transplantable cancers in mice. This immunity is evident when the tumor used gives a low, as well as when it gives a high percentage of takes.

[Reprinted from The Journal of Experimental Medicine, January 1, 1919, Vol. xxix, No. 1, pp. 31–34.]

EFFECT OF STIMULATION OF THE LYMPHOCYTES ON THE RATE OF GROWTH OF SPONTANEOUS TUMORS IN MICE.*

By JAMES B. MURPHY, M.D., and ERNEST STURM.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 21, 1918.)

Murphy and Morton[1] have shown that mice with spontaneous tumors when exposed to small doses of x-rays sufficient to stimulate somewhat the lymphocytes exhibit an increased resistance to replants of their own tumors. In these experiments the tumors were removed by operation and with the tumor out the animal was exposed to a suitable small dose of x-rays. Immediately afterwards a graft of the original tumor was reinoculated into the groin of the mouse. Another series of mice with spontaneous tumors from the same strain was submitted to the same procedure, except that the x-ray treatment was omitted. 50 per cent of the x-rayed mice showed no growth of the returned graft or local recurrence of the cancer. The other 50 per cent showed a growth of the graft which appeared at a much later period than in the untreated animals. In the control group of animals, in which the x-ray treatment was omitted, over 96 per cent of the animals showed a growth of the returned graft. The latter figure corresponds to that obtained by other observers with similar experiments.

In the work to be reported here we have used dry heat as the method of stimulation instead of x-rays. A mouse exposed for 5 minutes to dry heat at a temperature ranging from 55–63°C. will show at first a slight fall in the circulating lymphocytes, followed by a marked increase, lasting from 2 to 4 weeks.[2] Accompanying the increase in

* This investigation was carried out by means of funds from the Rutherford Donation.

[1] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.

[2] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 1.

the circulating lymphocytes there is a marked activity on the part of the lymphoid organs evidenced by numerous mitotic figures in the germinal centers.[3]

Experiment.—The animals for this experiment were procured from the Lathrop stock.[4] There were 61 mice showing numerous gradations and types of mammary carcinomas. The tumors were removed as completely as possible by operation, and while the tumor was out the animal was heated for 5 minutes over an electric heat lamp at a temperature of 55–63°C. Immediately after heating, a graft of the original tumor was reinoculated subcutaneously into the left groin of the animal. In 36 of the 61 animals so treated a complete immunity to recurrence of the growth of the graft resulted. There were no metastases observed in any of these 36 animals. Only those mice were included in the series which lived for a period of over 4 weeks and remained in good condition. The majority lived for a much longer period than this. Among the 25 animals of this series which were not immune, the average time for the graft to become large enough to be recognized was 2 weeks and 5 days. The number of recurrences was seven. In some instances there was a recurrence which later retrogressed, but these have not been recorded as immune animals.

For controls we used our former series of animals which were taken from the same stock and were treated in the same way except that they were not heated. Of twenty-nine animals the returned graft grew in twenty-eight, and the average time required for the graft to become palpable was 1 week and 5 days. Local recurrences were present in fourteen out of the twenty-nine animals. Table I gives a comparison of the two series.

TABLE I.

Series No.*	Immune.	Susceptible.	Local recurrences.	Grafts alone.	Questionable grafts.	Metastasis alone.	Time for appearance of graft.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
I	59.4	40.6	11.3	14.7	8.1	6.5	2 wks. and 5 days.
II	3.4	96.6	48.3	48.3			1 wk. " 5 "

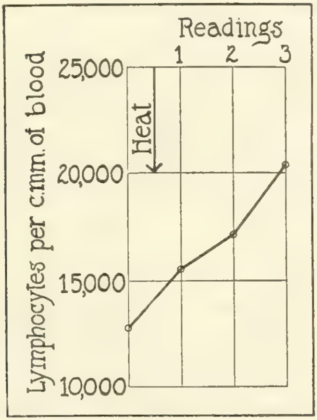
* Series I. 61 mice with spontaneous cancers heated after the removal of the tumor with later a return of a graft.

Series II. 29 control mice with cancer removed at operation and later a return of a graft.

[3] Nakahara, W., *J. Exp. Med.*, 1919, xxix, 17.

[4] The Lathrop stock of mice was recently purchased for The Rockefeller Institute for Medical Research by funds from the Rutherford Donation.

Blood counts were made on about half of the animals of the treated series, one before operation, and the next 1 week after operation and treatment, and subsequent counts were made at weekly intervals (Text-fig. 1). The average number of lymphocytes per c.mm. of blood before operation was about 12,000. 1 week after operation and heating they had risen to approximately 16,000, and they continued to increase until by the fourth count there were 20,000 lymphocytes per c.mm. of blood. In twenty of thirty-eight animals counted the polymorphonuclear leucocytes were lower after treatment than before. In the other eighteen, except in two or three cases where extensive infection occurred, there



Text-Fig. 1. The average number of lymphocytes of mice with spontaneous tumor after having been subjected to an exposure of heat. The counts were made before and at weekly intervals after heating.

was only a slight gain. These figures correspond with those reported for normal animals after exposure to heat. Counts made on untreated animals subjected to the same surgical procedure showed no such change.

DISCUSSION.

Pathologists from an early date have noted that lymphocytes accumulate about the slowly growing cancers, while they are absent in the more rapidly growing malignant types. This point has received a certain amount of comment but slight attempt has been

made at explanation. Our present results suggest that this infiltration may be of more importance than it has been previously considered. The great obstacle to overcome in accepting the lymphocyte as a factor of resistance to cancer growth is the fact that the lymph glands are the common point of metastases. This fact needs further study and elucidation but is not to be looked upon as an insurmountable barrier. Such possible questions present themselves as: What is the condition of the glands to which metastases take place? Is the resistance offered overcome by the number of cancer cells lodging in the lymph spaces? Are the lymphocytes in the gland in an actively functioning stage or do they require an activating substance like the opsonins? It is impossible to discuss these points with our present knowledge. The experiments reported here, coupled with the previous ones with x-rays, suggest strongly that the lymphoid tissue does offer a resisting influence to cancer growth, but this conception cannot be accepted unreservedly until further light is thrown on the activities of the lymph glands.

SUMMARY.

Spontaneous cancers were removed from a series of mice by operation. The animals were then subjected to an exposure to dry heat at a temperature ranging from 55–63°C. for 5 minutes. Immediately afterwards a graft of the original tumor was returned. The mice so treated exhibited a marked increase in their resistance to the growth of the cancer graft, over 59 per cent remaining entirely free from a return of the cancer. In a control series in which no treatment was given 96 per cent of the animals showed a return of the cancer.

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EXPERIMENTS ON THE RÔLE OF LYMPHOID TISSUE IN THE RESISTANCE TO EXPERIMENTAL TUBERCULOSIS IN MICE.

III. EFFECT OF HEAT ON RESISTANCE TO TUBERCULOSIS.

BY JAMES B. MURPHY, M.D., AND ERNEST STURM.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 19, 1918.)

In a previous report from this laboratory a series of experiments was recorded which seemed to explain the results of Lewis and Margot,[1] who noted that splenectomized mice had a greater resistance to tuberculosis than normal mice. Murphy and Ellis[2] demonstrated the fact that splenectomized mice, if exposed to suitable doses of x-rays no longer had an increased resistance, but were hypersusceptible to the infection. The interpretation of these results suggested, was that the increased resistance in splenectomized mice was due to the increase in the circulating lymphocytes, which was demonstrated to reach its height about 21 days after removal of the spleen. When the increase in the lymphocytes was prevented by x-rays, the increased resistance to tuberculosis was nullified. It was likewise shown that intact animals could be rendered less resistant to tuberculosis than controls by the use of broken doses of x-rays. The latter observation was confirmed by Morton[3] for the human strain of the organism with guinea pigs. Later Taylor and Murphy[4] showed that mice with a marked increase in the lymphocytes resulting from cancer inoculation in cancer-immune animals had also a marked increase in their resistance to tuberculosis. In this instance also if the increase in lymphocytes was de-

[1] Lewis, P. A., and Margot, A. G., *J. Exp. Med.*, 1914, xix, 187.

[2] Murphy, Jas. B., and Ellis, A. W. M., *J. Exp. Med.*, 1914, xx, 397.

[3] Morton, J. J., *J. Exp. Med.*, 1916, xxiv, 419.

[4] Taylor, H. D., and Murphy, Jas. B., *J. Exp. Med.*, 1917, xxv, 609.

stroyed by x-rays, the animals were rendered highly susceptible to infection.

We regarded these results, coupled with the deductions from observations of the blood count in men with this disease, and the histology of the lesion, as strong direct evidence that the lymphocytes play an important part in the resistance of the animal to tuberculosis. With the development of a new method of stimulating the lymphocytes,[5] namely that of intense dry heat, we have another opportunity to test this conception.

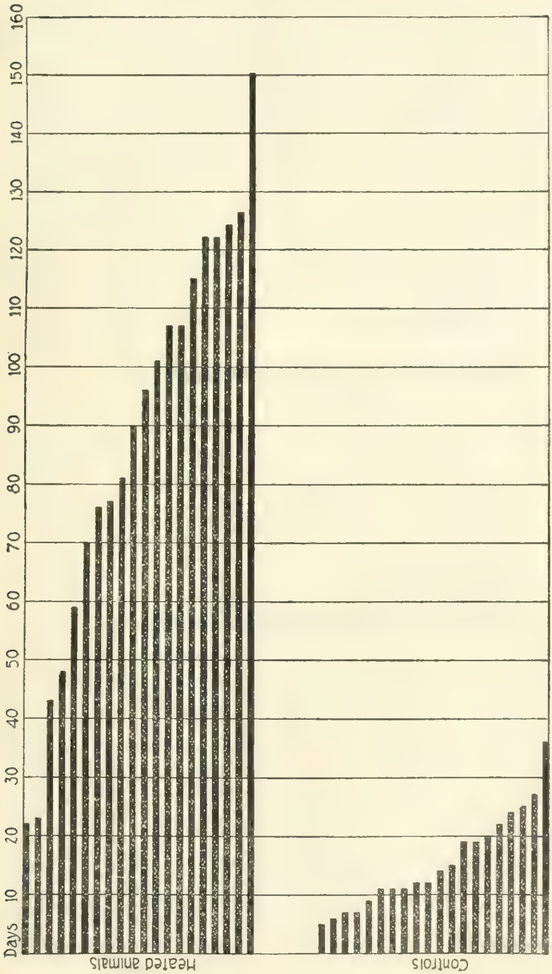
Method.

Mice subjected to dry heat ranging from 55–65°C. over a period of 5 minutes first show a fall in the total white blood count, both the polymorphonuclear cells and, to a lesser extent, the lymphocytes participating in the fall. The lymphocytes, however, show an immediate rebound, followed by a rapid increase which carries the count several hundred per cent above the normal. This rise continues for 14 to 21 days, after which there is a gradual return to the normal level. The polymorphonuclear leucocytes recover slowly and show no stimulation phase. This method gives us an excellent opportunity to test the effect of a marked increase in the lymphocytes on the course of tuberculous infections.

Experiment 1.—Forty mice of the same stock and of about the same age and size were selected for this experiment. Twenty were heated over an electric heat lamp for 5 minutes at a temperature starting at 55°C. and allowed to increase to 65°C. 1 week later these animals, together with the twenty controls, were each inoculated intraperitoneally with 2 mg. of a bovine strain of tubercle bacilli (4 week culture) suspended in 0.5 cc. of normal salt solution. The mice were then placed in individual jars in order to prevent the occurrence or spread of an epidemic. All the mice as they died were carefully autopsied, and films were taken from the peritoneal fluid, and from liver, kidney, lungs, and heart's blood. It was found that all the animals had widely disseminated tuberculosis, which could definitely be accepted as the cause of death.

The control animals died rapidly, the first one on the 6th day, and the last on the 36th day after inoculation. The average number of days of life for this group was approximately 16 days. None of the heated animals died until the

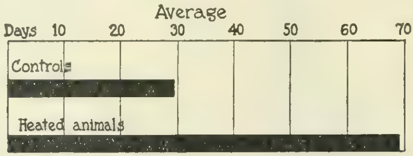
[5] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 1.



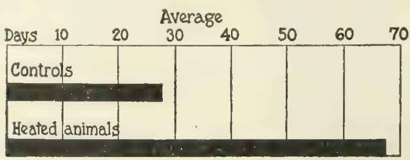
TEXT-FIG. 1. Each horizontal line represents the time of survival of a mouse after inoculation with bovine tubercle bacilli. The first group was subjected to an exposure of dry heat 1 week before inoculation. The second group was untreated.

22nd day after inoculation, and the longest period of life in this group was 150 days, the average length of life for the twenty mice being approximately 88 days. The rate at which the animals died is shown in Text-fig. 1.

Experiment 2.—Twelve healthy mice of about the same age and weight were heated in the same manner as in the previous experiment. A week later these, with twelve control mice from the same stock, were inoculated intraperitoneally with 2 mg. of a bovine strain of the tubercle bacillus suspended in 0.5 cc. of normal salt solution. The animals were segregated in individual jars as in the previous experiment. Autopsies were performed promptly after death and films taken from the principal organs, the peritoneal fluid, and the heart's blood.



TEXT-FIG. 2. The average duration of life of heated mice after inoculation with bovine tubercle bacilli compared with the duration of life of untreated animals. There were twelve mice in each group.



TEXT-FIG. 3. The average duration of life of heated mice after inoculation with bovine tubercle bacilli compared with the duration of life of untreated animals. There were twelve heated mice and fourteen controls.

All these mice showed extensive tuberculosis except one control which lived 89 days, and which was either a highly resistant animal or was not infected because of some accident in the inoculation. In this series the heated animals averaged 69 days of life after inoculation, while the controls averaged only 29 days. The details of this experiment are shown in Text-fig. 2.

Experiment 3.—Twenty-six mice of the same strain and about the same age were selected for the experiment. Twelve were subjected to direct heat ranging from 55–65°C. for 5 minutes. A week later each of the twenty-six animals was inoculated intraperitoneally with 2 mg. of a culture of the bovine tubercle bacillus suspended in 0.5 cc. of normal salt solution. They were segregated in separate

jars as in preceding experiments. Autopsies were performed as the animals died to verify the cause of death. In all, except one of the control animals, there was widely disseminated tuberculosis. In this animal, which lived 117 days, there was no evidence of the disease.

In this experiment the heated animals averaged 67.6 days of life after inoculation, while the controls lived only 27.8 days. The rate at which the mice died is shown in Text-fig. 3. In the last two experiments the same culture of the tubercle bacillus was used for inoculation.

The three experiments described show that animals subjected to one exposure of dry heat and inoculated a week later with a bovine strain of the tubercle bacillus virulent for mice had a greatly enhanced resistance to the organism as compared with that of the untreated animals. The difference in resistance here is much more striking than that seen in splenectomized animals or in the cancer-immune animals.

DISCUSSION.

The impression is gathered from the literature that individuals recovering from tuberculosis develop an increase in the number of circulating lymphocytes, although the point is rarely emphasized. In an analysis of a number of blood counts on rapidly advanced cases of tuberculosis in man a marked decrease in this type of cell is observed. The fact that between these two characteristic findings there is every variation in the type and degree of reaction, with often practically normal blood counts, has led to skepticism in regard to the significance of the blood changes in tuberculosis. In dealing with such a slow, chronic infection, the changes would not be expected in a marked degree except in the two extremes mentioned above. The blood changes, together with the fact that the tuberculous lesion is characterized by an accumulation of large numbers of lymphocytes, are suggestive of the part played by this cell in resistance to tuberculosis. To this evidence we add the results of our experiments; namely, the lowering of resistance to tuberculosis in animals depleted of their lymphocytes by means of x-rays, and the increased resistance in animals with the lymphocytes increased by three widely different methods, splenectomy, cancer immunity, and dry heat.

The chief points which may be brought against this conception is that tuberculosis frequently involves the lymphoid structures, and

they seem, if anything, more susceptible to the infection than many other tissues. The explanation of this fact is not clear, but we do not consider it an overwhelming argument in the face of the mass of evidence which indicates that the lymphocyte is an important factor in the resistance to the tubercle bacillus.

SUMMARY.

Mice with high lymphocyte counts and increased activity of the lymphoid tissue induced by one exposure to intense dry heat exhibit a marked increase in the resistance to large doses of bovine tubercle bacilli as compared with that shown by control animals given a similar inoculation. This resistance, judged by the time of survival after inoculation, is increased from two- to threefold. The average length of life after inoculation for three groups of heated mice was 88, 69, and 67 days respectively, while the control groups averaged 16, 29, and 28 days respectively.

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EFFECT OF EXPOSURE TO THE SUN ON THE CIRCULATING LYMPHOCYTES IN MAN.

BY HERBERT D. TAYLOR, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 12, 1918.)

Experiments reported previously[1-4] indicate that in animals a blood lymphocytosis attends increased resistance to cancer and tuberculosis. The work has shown that the number of circulating lymphocytes can be varied by suitable dosage of the Roentgen rays. Massive x-ray exposures decrease, while small ones increase, both proportionately and actually, the number of lymphocytes in the circulating blood. It seemed possible that the beneficial results of heliotherapy in surgical tuberculosis noted by Rollier and others[5] might be due, in part at least, to a similar effect of the actinic rays on the lymphoid organs, leading to an increase in the number of circulating lymphocytes. The fact that heliotherapy is applied chiefly at high altitudes in the Alps suggested also that the actinic rather than the heat rays were the therapeutic agents. The ultraviolet rays contained in light of solar origin lie in that portion of the invisible spectrum included between 4,000 and 2,950 Ångström units.[6] Light waves of lengths included between 2,100 and 2,800 and, to a lesser extent, those up to 4,000 Ångström units are bactericidal.[7] However, as shown by Newcomer as well as by previous observers, this action is lost in the presence of organic matter in minute traces. Inasmuch as the surgical lesions treated successfully in this manner

[1] Murphy, Jas. B., and Ellis, A. W. M., *J. Exp. Med.*, 1914, xx, 397.

[2] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

[3] Morton, J. J., *J. Exp. Med.*, 1916, xxiv, 419.

[4] Taylor, H. D., and Murphy, Jas. B., *J. Exp. Med.*, 1917, xxv, 609.

[5] For a review of the subject see: Roatta, G. B., L'Elioterapia nella pratica medica e nell 'educazione, Milan, 1914.

[6] Bovie, W. T., *Bot. Gaz.*, 1915, lix, 149.

[7] Newcomer, H. S., *J. Exp. Med.*, 1917, xxvi, 841.

are deep seated and the ultra-violet rays have but feeble penetrating power, it does not seem probable that a direct destructive effect on their bacterial causes is responsible for the clinical improvement reported. Fresh air, good food, and rest, in the absence of sunlight do not produce a corresponding effect.

In order to determine whether exposure to the sun has any effect on the circulating lymphocytes of healthy individuals, the following study was made.

A series of blood counts was made, in the spring of 1916, on several individuals who expected to be exposed to the sun during the ensuing summer months at Woods Hole, Massachusetts. In the fall a second series of counts was made on the same subjects, and this report is based on observations made on the bloods of 38 persons who showed more or less evidence of chronic solar dermatitis (tanning) at the time the final blood count was made. Most of the individuals studied were tanned over a large portion of their bodies, and no instances are included in which there was tanning merely of the face and hands. The subjects included both sexes and ranged in age from 20 to 65 years. It is admitted that tanning of the skin is caused by actinic and not by heat rays.

Method.

The blood to be counted, obtained from a needle prick in the tip of the finger, was diluted 1:20 with 3 per cent acetic acid solution, the mixture shaken in a diluting pipette for 5 minutes, a drop placed on a Türck hemocytometer, and an interval allowed for the corpuscles to settle. The white corpuscles in 8 of the 9 ruled millimeter squares of the counting chamber were counted and the number per c.mm. was estimated in the usual way. Films made at the same time on cover-slips were used for differential leucocyte counts. After staining with Wright's stain, 300 cells were counted and the percentage of each type was determined. The total number of lymphocytes per c.mm. of blood was estimated by multiplying the total number of white corpuscles per c.mm. by the percentage as determined by differential count.

The results of the study, for convenience arranged in Tables I to VIII, are analyzed in the discussion which follows.

Discussion of Tables.—A study of the tables brings out the fact that the blood of persons in whom chronic solar dermatitis is present shows not infrequently considerable percentage and actual increase of the lymphocytes. Thus in 25, or 65.8 per cent of the 38 persons

TABLE I.

Individuals Showing an Increase in Lymphocytes after Exposure to Sun.

Individual No.	Lymphocytes.		Increase.
	Before exposure to sun.	After exposure to sun.	
	per cent	*per cent*	*per cent*
1	28.1	43.8	55.5
2	26.3	31.7	20.8
3	33.0	39.0	18.1
4	19.6	25.3	29.0
5	30.0	32.0	8.6
6	26.0	36.0	38.5
7	28.0	34.0	21.4
8	22.6	32.6	44.2
9	35.0	42.6	21.7
10	30.3	37.0	22.1
11	38.6	47.3	22.5
12	20.0	22.0	10.0
13	22.6	29.6	30.9
14	26.0	35.0	36.9
15	19.6	42.2	115.3
16	33.6	47.2	40.5
17	27.1	33.0	21.8
18	22.0	25.6	16.4
19	28.0	33.6	20.0
20	23.6	25.9	9.7
21	30.0	42.8	42.7
22	28.0	31.8	13.6
23	34.6	39.0	12.7
24	26.0	42.4	63.1
25	27.0	39.6	46.6
Average...........	27.4	35.6	27.2

studied, there was a definite increase in the number of circulating lymphocytes after development of solar pigmentation which averaged 27.2 per cent. This increase, given in the column headed "Increase" in Table I, represents the percentile rise of lymphocytes above the

TABLE II.

Individuals Showing a Decrease in Lymphocytes after Exposure to Sun.

Individual No.	Lymphocytes.		Decrease.
	Before exposure to sun.	After exposure to sun.	
	per cent	*per cent*	*per cent*
26	34.6	32.2	6.9
27	42.3	33.0	21.3
28	35.5	28.0	21.1
29	41.3	29.1	29.5
30	42.0	36.3	13.6
31	42.3	37.6	11.1
32	43.8	42.7	2.5
33	34.6	32.6	5.8
Average...........	39.6	33.9	15.2

TABLE III.

Individuals Showing No Change in Lymphocyte Percentage after Exposure to Sun.

Individual No.	Lymphocytes.		Increase.	Decrease.
	Before exposure to sun.	After exposure to sun.		
	per cent	*per cent*	*per cent*	*per cent*
34	29.3	30.0	2.4	
35	33.3	33.0		0.9
36	18.0	17.6		2.2
37	27.5	26.9		2.2
38	27.0	27.8	2.9	
Average.......	27.0	27.0	5.3	5.3

TABLE IV.

Summary of Bloods Studied.

Individuals with.	No.	Per cent.
Lymphocytes increased after exposure to sun..................	25	65.8
" decreased " " " "	8	21.0
" showing no change after exposure to sun...........	5	13.2
Total...	38	100.0

original percentage of these cells. In eight, or 21 per cent, there was an average decrease in the percentage of lymphocytes amounting to 15.2 per cent (Table II). In five, or 13 per cent, there was no substantial change in the lymphocyte percentage (Table III).

If one disregards for the moment the individuals, 5 in number, whose blood showed no definite percentage change, it follows that of the remaining 33, 25, or 73.8 per cent, exhibit an increase, and 8, or 24.2 per cent, a decrease of the circulating lymphocytes. The changes noted in the number of total lymphocytes before and after tanning tended to parallel the percentage change in the same blood. Thus (Table V) twenty-five bloods which gave a definite percentage lymphocyte increase gave also an average absolute increase from 2,574 to 3,338 cells per c.mm. Moreover, the eight bloods which

TABLE V.

Average Total Lymphocyte Counts.

Individuals (Tables I, II, and III.)	Average before exposure to sun.	Average after exposure to sun.	Increase.	Decrease.
			per cent	*per cent*
25 with percentage increase................	2,574	3,338	29.6	
8 " " decrease.................	3,807	3,187		16.3
5 " no change in percentage............	2,951	3,060	3.5	

gave a decrease in the percentage of lymphocytes followed the same trend, as the average number of these cells before exposure was 3,807, and after 3,187 per c.mm. It should be noted here that the bloods showing no appreciable change in percentage of lymphocytes gave an average total number per c.mm. which varied only slightly as the result of the exposure, the average before exposure being 2,951 and after exposure 3,060.

The average percentage change in the total lymphocytes per c.mm., after exposure, for each of the three series compares closely with similar determinations of the percentile increase or decrease as given in Tables I, II, and III. The twenty-five persons who showed an average percentile increase of 27.2 showed a total lymphocyte increase of 29.6 per cent. The percentile decrease in the eight individuals included in Table II was 15.2, and the average total lymphocyte

TABLE VI.

Complete Count on Individuals Showing an Increase in Lymphocytes after Sunburn.

Individual No.	White blood corpuscles.	Small lymphocytes.	Large lymphocytes.	Polymorphonuclear neutrophils.	Polymorphonuclear eosinophils.	Polymorphonuclear basophils.	Transitionals and large mononuclears.	Lymphocytes.	Absolute lymphocytes.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
1	8,648*†	24.2	3.8	66.1	3.2	0.1	2.4	28.1	2,377
	6,850	40.8	3.0	48.0	3.5	0.8	3.8	43.8	2,978
2	7,720	20.6	5.7	65.5	1.7	1.3	5.2	26.3	2,048
	8,550	27.7	3.8	60.4	3.1	0.6	3.9	31.7	2,725
3	9,710	28.5	4.5	59.8	2.0	0.8	4.3	33.0	3,105
	6,920	34.0	5.0	58.0	0.6	0.3	2.0	39.0	2,739
4	13,450	15.6	4.0	76.3	0.0	0.3	3.6	19.6	2,636
	10,900	23.3	2.0	67.3	3.6	0.3	3.3	25.3	2,758
5	7,390	26.5	3.5	65.0	1.0	3.8	0.3	30.0	2,213
	8,240	27.6	5.0	65.0	0.3	0.0	1.6	32.6	2,687
6	15,900	22.3	3.6	69.3	2.3	0.0	2.3	26.0	4,134
	7,159	32.8	3.5	53.7	1.7	0.3	8.0	36.0	2,675
7	9,650	25.3	2.6	62.0	3.0	0.0	7.0	28.0	2,702
	7,100	29.3	4.7	57.5	4.2	0.2	4.2	34.0	2,419
8	10,750	20.0	2.6	75.3	0.0	0.3	1.6	22.6	2,430
	9,750	31.0	1.6	63.0	1.6	0.3	2.3	32.6	3,180
9	7,250	31.3	3.6	56.0	4.0	0.6	4.3	35.0	2,538
	5,050	40.0	2.6	46.0	4.6	0.0	6.6	42.6	2,151
10	14,750	26.6	3.6	57.3	8.3	1.3	2.6	30.3	4,469
	8,875	34.3	2.7	53.8	4.8	0.8	3.5	37.0	3,330
11	8,250	37.0	1.6	55.6	2.6	0.0	3.0	38.6	3,185
	11,750	43.3	4.0	46.6	1.3	0.0	4.6	47.3	5,558
12	9,700	16.6	3.3	77.3	0.3	1.3	1.0	20.0	1,940
	11,850	21.3	0.6	68.6	5.0	0.3	4.0	22.0	2,607

*Average counts are given in Tables VI to VIII because in many instances from two to six counts were made before, and a similar number after exposure to the sun.

†The upper row of figures for each individual represents counts before, and the lower row counts after exposure to the sun.

TABLE VI—*Concluded.*

Individual No.	White blood corpuscles.	Small lymphocytes.	Large lymphocytes.	Polymorphonuclear neutrophils.	Polymorphonuclear eosinophils.	Polymorphonuclear basophils.	Transitionals and large mononuclears.	Lymphocytes.	Absolute lymphocytes.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
13	10,667	20.6	2.0	67.0	9.3	0.3	0.6	22.6	2,410
	14,500	25.3	4.3	59.3	5.3	0.3	5.3	29.6	4,292
14	7,800	23.0	3.0	59.0	1.6	1.0	2.3	26.0	2,028
	9,500	33.3	2.3	61.0	2.3	0.0	1.0	35.6	3,382
15	7,050	16.3	3.3	74.6	0.0	0.3	5.3	19.6	1,382
	6,200	39.6	2.6	52.0	0.0	0.6	5.0	42.2	2,616
16	6,000	29.0	4.6	58.6	3.0	1.0	3.6	33.6	2,016
	5,800	42.6	4.6	46.0	1.0	1.6	4.0	47.2	2,738
17	7,925	24.6	2.5	68.3	0.8	0.2	3.5	27.1	2,189
	9,600	30.3	2.6	60.3	2.0	0.3	4.3	33.0	3,168
18	6,700	17.5	4.5	70.0	4.0	0.0	4.0	22.0	1,474
	7,350	20.6	5.0	64.6	2.6	0.0	7.0	25.6	1,882
19	9,300	25.6	2.3	59.3	10.0	0.6	2.0	28.0	2,604
	11,100	32.6	1.0	59.6	3.3	0.3	3.0	33.6	3,696
20	6,850	21.0	2.6	72.0	0.6	0.3	3.3	23.6	1,617
	7,825	22.8	3.1	68.3	2.0	0.2	3.5	25.9	1,983
21	10,850	26.3	3.6	62.6	3.3	0.6	3.3	30.0	3,255
	9,550	40.3	2.5	47.3	6.0	0.5	3.3	42.8	3,886
22	9,100	25.6	2.3	65.6	2.3	0.0	4.0	28.0	2,548
	9,900	30.6	1.1	62.8	2.3	0.2	2.8	31.8	3,084
23	8,200	32.3	2.3	60.0	1.0	0.0	4.3	34.6	2,837
	8,900	35.6	3.3	53.3	2.0	1.0	4.6	39.0	3,471
24	12,778	25.0	1.0	69.6	1.6	0.0	2.6	26.0	3,322
	9,575	40.8	1.6	51.5	4.2	1.3	0.5	42.4	4,059
25	10,667	25.3	1.6	70.6	0.0	0.6	1.6	27.0	2,880
	12,550	38.3	1.3	56.3	1.0	1.0	2.0	39.6	4,970
Average....	9,482	23.9	3.1	65.7	2.6	0.6	3.1	27.4	2,574
	9,054	32.7	2.9	57.2	2.8	0.4	3.8	35.7	3,338

decrease was 16.3 per cent. Table III gives 0.0 as the average percentile change in the five persons included there, while the average total lymphocyte increase in the same group was 3.5 per cent.

TABLE VII.

Complete Counts on Individuals Showing a Decrease in Lymphocytes after Sunburn.

Individual No.	White blood corpuscles.	Small lymphocytes.	Large lymphocytes.	Polymorphonuclear neutrophils.	Polymorphonuclear eosinophils.	Polymorphonuclear basophils.	Transitionals and large mononuclears.	Lymphocytes.	Absolute lymphocytes.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
26	8,683	29.9	4.8	58.9	0.6	0.7	5.0	34.6	2,973
	8,933	28.8	3.4	62.0	1.1	0.5	4.1	32.2	2,768
27	6,714	36.8	5.5	46.5	2.3	1.8	7.0	42.3	2,874
	7,400	30.3	2.6	62.6	0.0	1.0	3.3	32.0	2,442
28	6,249	30.5	5.0	58.6	1.3	0.4	4.2	35.5	2,199
	8,658	23.8	4.3	64.6	2.2	0.6	4.5	28.0	2,353
29	7,900	37.0	4.3	49.6	1.6	0.3	7.0	41.3	3,263
	10,250	26.3	3.2	59.5	3.0	0.6	7.2	29.1	2,972
30	12,750	37.0	5.0	55.0	0.6	0.3	2.0	42.0	5,355
	13,000	34.0	2.3	59.3	1.0	0.3	3.0	36.3	4,719
31	11,100	39.6	2.6	53.3	0.6	0.0	1.6	42.3	4,695
	9,050	36.0	1.6	56.6	0.0	0.3	5.3	37.6	3,403
32	6,850	39.5	4.3	47.8	1.8	1.2	5.3	43.8	3,000
	6,783	40.5	2.2	51.2	2.9	0.4	2.6	42.7	2,917
33	8,950	31.6	3.0	57.6	3.3	0.0	4.3	34.6	3,097
	8,950	28.3	4.3	63.0	2.3	0.0	2.0	32.6	2,918
Average....	8,650	35.2	4.3	53.4	1.5	0.6	4.6	39.6	3,807
	9,128	31.0	3.0	59.9	1.7	0.5	4.0	33.8	3,187

As may be seen from Tables VI, VII, and VIII there was no constant change in the total numbers of white blood corpuscles per c.mm. of blood as determined before and after exposure. A study of these tables also shows that the polymorphonuclear neutrophilic leucocytes tended to vary in inverse proportion with the lympho-

cytes, these cells decreasing when the lymphocytes increased and *vice versa*. The polymorphonuclear eosinophilic and basophilic (mast) cells and the group composed of the transitional and large mononuclear granular cells (bone marrow oxidase cells, Evans[8]) remained very constant.

TABLE VIII.

Complete Counts on Individuals Showing No Change in Lymphocytes after Sunburn.

Individual No.	White blood corpuscles.	Small lymphocytes.	Large lymphocytes.	Polymorphonuclear neutrophils.	Polymorphonuclear eosinophils.	Polymorphonuclear basophils.	Transitionals and large mononuclears.	Lymphocytes.	Absolute lymphocytes.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
34	15,600	26.3	3.0	63.6	1.0	1.0	5.0	29.3	4,571
	17,800	25.3	4.6	61.6	1.6	0.6	6.0	30.0	5,340
35	7,900	28.3	5.0	61.6	0.6	0.3	4.0	33.3	2,631
	7,850	31.0	2.0	61.3	1.3	0.6	3.6	33.0	2,591
36	11,350	14.3	3.6	77.0	2.0	1.0	2.0	18.0	2,043
	13,950	15.3	2.3	74.3	0.6	0.6	6.6	17.6	2,455
37	11,975	24.9	2.5	68.3	0.5	0.8	2.8	27.5	3,378
	9,750	24.7	2.1	67.4	2.4	0.5	2.6	26.9	2,600
38	7,900	24.3	2.6	61.6	6.0	0.0	5.3	27.0	2,133
	7,825	23.3	4.5	62.2	5.0	0.5	4.5	27.8	2,213
Average....	10,945	23.6	3.4	66.4	2.0	0.6	3.8	27.0	2,951
	11,435	23.9	3.1	65.4	2.2	0.6	4.7	27.1	3,060

Rollier and others[9] have emphasized the fact that tanning is necessary to obtain beneficial results in tuberculosis with heliotherapy. In this connection it is interesting to note that of the thirteen individuals who showed no increase in their circulating lymphocytes, six had the pasty type of skin which does not tan. Three others were so dark normally that it was impossible to determine whether or not there had been additional pigmentation. Five

[8] Evans, F. A., *Arch. Int. Med.*, 1916, xviii, 692.

[9] Rollier, A., etc., quoted in Roatta,[5] p. 19.

of the eight persons whose blood showed a loss in lymphocyte percentage had circulating cells of this type in excess of 40 per cent before exposure to the sun. These observations are interesting as offering possible explanations of the apparent exceptions to the general tendency to develop a definite lymphocytosis after chronic solar dermatitis. Thus the twenty-five persons with a definite increase in circulating lymphocytes were all tanned to a greater or less extent. The increase in lymphocytes, moreover, tended to be greatest in the individuals in whom the pigmentation was most increased, yet this was not invariably the case; so that the increase in lymphocytes and degree of tanning did not always go hand in hand so far as this series of tests is concerned.

In regard to complexion, no particular difference between the blood or skin response to solar irritation was noted with the exception of the three individuals in whom it was impossible to determine whether or not additional pigmentation was present. So far as observed age and sex had no effect on the blood response to light.

DISCUSSION.

From the results recorded here it seems that the rays contained in the solar spectrum, as it reaches the earth in the temperate zone in midsummer, bring about changes in the white corpuscles of the blood in a manner similar to that recognized as characteristic of the x-rays. The manner of action is equally obscure with regard to both agents.

Of interest in connection with this study are blood counts made by Chamberlain and Vedder[10] on Americans living in the Philippines. Although analyzed by them to ascertain the effect of the tropical climate on the Arneth blood picture, their counts show a higher percentage of lymphocytes than normal individuals in the temperate zone.[11] In the 72 cases reported there was an average of 38.6 per cent. The tropical service of the soldiers composing the series averaged 28.7

[10] Chamberlain, W. P., and Vedder, E. B., *Philippine. J. Sc.*, 1911, vi, 408.

[11] In a series of more than 100 observations on the bloods of 46 healthy individuals from 20 to 65 years of age, made in connection with this study, it was found that the lymphocytes averaged 29.3 per cent.

months. Wickline,[12] in counts made upon the blood of 104 soldiers 3, 12, and 19 months after arrival in the Philippines, showed that there was a definite and progressive increase in the mononuclear cells. These cells averaged 31.4 per cent at the first, 34.4 per cent at the second, and 39.5 per cent at the third determination. Although there is no distinction made between the mononuclear elements, tending to separate the lymphocytes from the cells belonging to the granular series, some idea of the direction in which the cells of lymphatic origin varied may be obtained by following the percentage of small mononuclear cells, which are undoubtedly the small lymphocytes. The average percentage of these cells at the first count was 21.8, at the second count 26.6, and at the third count 33.3. Disregarding the large lymphocytes, which should be combined with the small cells of the same series to give an accurate idea of their number at each determination, it can be seen that there was a definite increase of these cells in the blood after 19 months in the tropical climate. The soldiers were undoubtedly exposed to the sun for long periods of time. It also seems possible that the invisible spectrum is somewhat widened in the tropics[13] and there is consequently a wider range of ultra-violet rays than in the temperate zone. The intensity of the visible rays of the spectrum is also increased as we approach the equator. It is probable that the invisible rays increase likewise in proportion to those that are visible. Although the effect on the blood seems to be related to the ultra-violet rays of sunlight it is impossible to rule out the effect of infra-red, or heat waves in these observations.

SUMMARY.

Chronic solar dermatitis was accompanied, in 25 of the 38 individuals studied, by an appreciable increase, percentage and absolute, in the number of circulating lymphocytes.

In eight there was a definite decrease and in five no appreciable change after prolonged exposure to the rays of the summer sun.

Of the thirteen subjects with no increase in blood lymphocytes, six

[12] Wickline, W. A., *Mil. Surg.*, 1908, xxiii, 282.

[13] Bovie, W. T., *Am. J. Trop. Dis. and Prev. Med.*, 1914–15, ii, 512.

failed to tan, three were so dark originally that to determine an increase was impossible, and five had an extremely high lymphocyte count at the first count.

Blood counts on white persons living in the Philippines indicate that the blood lymphocytes are likewise increased after a prolonged period of residence in the tropical zone.

Because of the parallelism between the tanning and the blood changes it seems probable that the lymphocytosis observed in the majority of instances, which is similar to the response of the blood of animals to small doses of the x-rays, is due to the effects of the ultra-violet rays contained in the solar spectrum.

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STUDIES ON X-RAY EFFECTS.

I. Destructive Action on Blood Cells.*

By HERBERT D. TAYLOR, M.D., WILLIAM D. WITHERBEE, M.D., and JAMES B. MURPHY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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Pusey,[1] Senn,[2] Brown,[3] Bryant and Crane,[4] and others,[5,6] have reported on cases of leucemia treated with the x-rays and all seem to agree that there is a definite reduction of the circulating leucocytes, following treatment. Most of the cases reported were, however, of the splenomyelogenous type and the myelocytes were the cells most affected. Capps and Smith[7] treated lymphatic leucemia with the Roentgen rays and noted a marked reduction of the circulating lymphocytes, after treatment, in five of their six cases.

Heineke,[8] the first to make careful histological examinations of animals following x-ray exposures, demonstrated that the lymphatic tissues of the body were primarily affected. He found degeneration of the lymph follicles in the spleen and lymph glands and a diminution of circulating lymphocytes. He correlated his observations by suggesting that the diminution in circulating lymphocytes is directly referable to the selective destruction of the lymphogenic tissues by the x-rays.

Helber and Linser[9] published blood counts made on a rabbit before and after x-ray treatment. A marked percentile and absolute reduction in the circulating

* This investigation was carried out by means of funds from the Rutherford Donation.

[1] Pusey, W. A., *J. Am. Med. Assn.*, 1902, xxxviii, 166.

[2] Senn, N., *N. Y. Med. J.*, 1903, lxxvii, 665.

[3] Brown, E. J., *J. Am. Med. Assn.*, 1904, xlii, 827.

[4] Bryant, B. L., and Crane, H. H., *Med. Rec.*, 1904, lxv, 574.

[5] For a review of the literature see Warthin, A. S., *Internat. Clin.*, 1906, iv, series 15, 243.

[6] For a further review of the literature see Pancoast, H. K., *Univ. Penn. Med. Bull.*, 1906–07, xix, 282.

[7] Capps, J. A., and Smith, J. F., *J. Am. Med. Assn.*, 1904, xliii, 981.

[8] Heineke, H., *Mitt. Grenzgeb. Med. u. Chir.*, 1905, xiv, 21.

[9] Helber, E., and Linser, P., *Münch. med. Woch.*, 1905, lii, 689.
Linser, P., and Helber, E., *Deutsch. Arch. klin. Med.*, 1905, lxxxiii, 479.

lymphocytes occurred. These cells, before treatment, represented 32 per cent of the white blood corpuscles, a total of 2,080 lymphocytes per c.mm. of blood. After treatment they had decreased to 6 per cent, or but 120 cells per c. mm. In a white rat there was a reduction of lymphocytes from 60 per cent, or 8,400 cells before, to 30 per cent, or 1,020 cells after x-ray treatment. These results were confirmed by other experiments.

Warthin[5] confirmed Heineke's work and added further evidence indicating that the x-rays have a specific destructive action on lymphoid tissues. He found the Malpighian bodies of the spleen to be first affected and later the lymph glands and bone marrow. Histologically, he noted fragmentation of the tissue lymphocytes, the particles being ingested by phagocytes. Mitotic figures were absent or very infrequently encountered in the lymphogenic tissues following x-ray treatment. After a short interval the lymph follicles, when present, were either inconspicuous or invisible in gross, and but few lymphocytic elements were found microscopically. The stroma of the organs affected was much in evidence, owing to the destruction and consequent disappearance of the lymphogenic cells. Heineke and Warthin both employed x-rays in lethal doses.

It has been shown that resistance to cancer[10] and tuberculosis,[11] in animals, is attended by an increase in number of the circulating lymphocytes and that the resistance, as well as the lymphocytosis, may be destroyed by x-rays in proper dosage.[11, 12] Likewise the natural resistance of animals to heteroplastic tissue grafts, which seems to be associated definitely with a local accumulation of lymphocytes, may be destroyed by the x-rays.[13]

Inasmuch as (*a*) it is not possible to estimate the dosage employed in most of the older experiments concerning the effect of the x-rays on the blood, because gas tubes were used, (*b*) only a few blood counts have been published in these cases and their significance has not been adequately explained, (*c*) the x-rays are now being used with increasing frequency in therapeusis; it seemed important to obtain accurate information regarding the response of the blood to x-rays, and with this purpose the following results are recorded.

EXPERIMENTAL.

Experiment 1.—Eight areas, comprising both flanks of a Shetland pony, about 8 years old, were successively exposed in a single day to unfiltered x-rays generated by a Coolidge tube. The factors governing the dose at each exposure were:

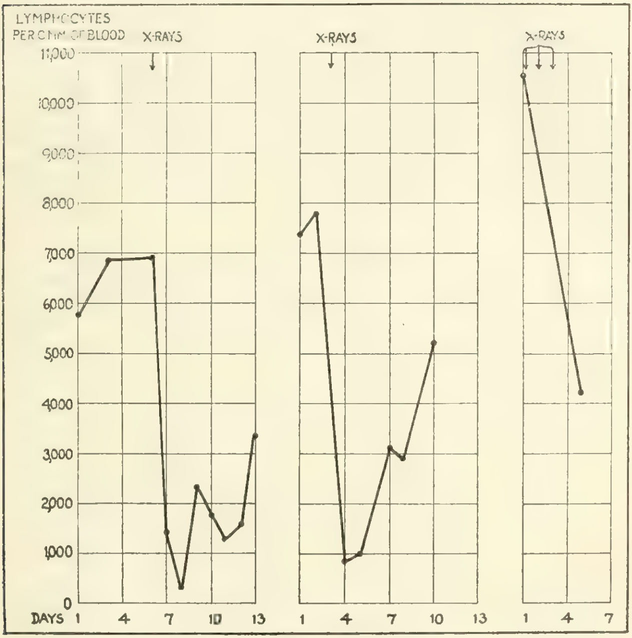
[10] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

[11] Taylor, H. D., and Murphy, Jas. B., *J. Exp. Med.*, 1917, xxv, 609.

[12] Murphy, Jas. B., and Ellis, A. W. M., *J. Exp. Med.*, 1914, xx, 397.

[13] Murphy, Jas. B., *J. Am. Med. Assn.*, 1914, lxii, 1459.

spark-gap 8 inches, milliamperes 5, distance from the target to the skin 12 inches, and time 10 minutes. By computing the dose[14] from the factors given, it was found that the animal received over each area 20, or over the eight areas 160 Holzknecht



a b c

TEXT-FIG. 1, *a*, *b*, and *c*. (*a*) The effect of x-ray treatment on the circulating lymphocytes of a pony. (*b*) The effect of x-ray treatment on the circulating lymphocytes of a cat. (*c*) The effect of x-ray treatment on the circulating lymphocytes of a guinea pig.

units. The total number of lymphocytes per c. mm. of blood at each observation, during the period of study, is charted in Text-fig. 1, *a*. The per cent

[14] Remer, J., and Witherbee, W. D., *Am. J. Roentgen.*, 1917, iv, 303.

and total number of lymphocytes and polymorphonuclear neutrophilic leucocytes per c. mm. of blood at each determination are tabulated in Table I.

Experiment 2.—A healthy adult monkey (*Macacus rhesus*) was given a series of unfiltered x-ray treatments, extending over a period of 8 days. Seven doses were given, the dorsal and ventral surfaces of the body being alternately exposed. The factors at each treatment were: spark-gap 3 inches, milliamperes 10, distance from the target of the Coolidge tube to the skin 12 inches, and time 4 minutes. This dose represents, at each exposure 6, or a total of 42 Holzknecht units. Text-fig. 2 graphically illustrates the effect of the x-rays on the circulating lymphocytes in this animal, each determination referring to the total number of lymphocytes per c. mm. of blood. In Table II the blood counts, made at different times in the course of the experiment, are summarized to show more fully the changes in the circulating cells following treatment.

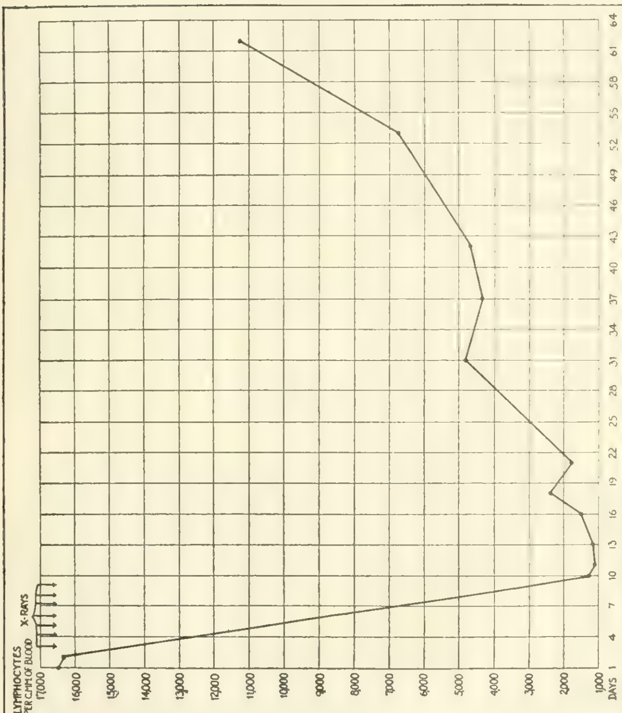
TABLE I.

Pony Receiving 160 Holzknecht Units.

Day of experiment.	Length of time after x-rays.	Lymphocytes.		Polymorphonuclear cells.	
		Per cent.	Total No.	Per cent.	Total No.
	days				
1		43.7	5,790	55.7	7,380
3		48.3	6,859	48.3	6,859
6		44.2	6,906	50.0	7,813
7	1	6.3	1,473	93.7	21,902
8	2	1.2	321	92.7	24,797
9	3	11.7	2,404	84.7	17,406
10	4	24.2	1,785	66.7	4,918
11	5	29.3	1,282	60.3	2,638
12	6	27.0	1,593	66.3	3,912
13	7	31.6	3,410	64.0	6,912

Experiment 3.—A full grown cat was given two treatments in a single day with unfiltered x-rays emitted by a Coolidge tube. One exposure was made on the dorsal and one on the ventral surface of the body. The factors at each exposure were: spark-gap 3 inches, milliamperes 10, distance from the target to the skin 12 inches, and time 1 minute. The dose, in this instance, was for each exposure $1\frac{1}{2}$, or a total of 3 Holzknecht units. Text-fig. 1, *b* is a curve showing the total number of lymphocytes plotted against the days on which blood counts were made. Table III gives percentages and actual numbers of white blood cells on the days recorded in Text-fig. 1, *b*.

Experiment 4.—A guinea pig was exposed to unfiltered x-rays on 3 successive days, after an initial blood count had been made. On the 5th day, 48 hours after the last x-ray treatment, a second count was made; Text-fig. 1, *c* shows



TEXT-FIG. 2. The effect of x-ray treatment on the circulating lymphocytes of a *Macacus rhesus*.

graphically the fall in the total number of lymphocytes per c. mm. of blood following the administration of the x-rays. Table IV gives the actual figures determined at the two blood examinations. The factors at each treatment were:

TABLE II.

Monkey Receiving 42 Holzknecht Units.

Day of experiment.	Length of time after x-rays.	Lymphocytes.		Polymorphonuclear cells.	
		Per cent.	Total No.	Per cent.	Total No.
	days				
1		62.7	16,537	36.0	9,495
2		56.7	16,415	40.7	11,783
10	1	4.0	1,283	94.7	30,375
11	2	4.7	1,128	95.0	22,800
13	4	7.0	1,155	92.7	15,296
16	7	5.7	1,526	94.0	25,169
18	9	11.7	2,463	88.0	18,524
21	12	7.0	1,787	92.3	23,560
28	19	27.0	4,846	71.0	12,745
37	28	23.3	4,351	74.7	13,950
42	33	31.7	4,771	64.3	9,677
53	44	29.0	6,779	66.7	15,598
63	54	55.7	11,224	40.7	8,201

TABLE III.

Cat Receiving 3 Holzknecht Units.

Day of experiment.	Length of time after x-rays.	Lymphocytes.		Polymorphonuclear cells.	
		Per cent.	Total No.	Per cent.	Total No.
	days				
1		35.0	7,389	55.7	11,759
2		34.7	7,825	56.0	12,628
4	2	5.0	844	94.0	15,876
5	3	8.7	1,005	83.7	9,672
7	5	16.0	3,093	77.0	14,886
8	6	12.0	2,907	79.0	19,144
10	8	11.0	5,194	88.0	41,555*

* Distemper developed later in this cat and the high polymorphonuclear leucocyte count may be due to this.

spark-gap $2\frac{1}{4}$ inches, milliamperes 10, distance from the target of the Coolidge tube to the skin 12 inches, and time 3 minutes. This dose represents, approximately, 3 Holzknecht units.

In two experiments on mice a gas tube had been used to generate x-rays. The dose was necessarily indefinite and the only measure of the comparative amount of the x-rays received by each animal consists in the constant established by the fact that the mice included in each experiment were exposed simultaneously and for the same length of time. These experiments are included because they demonstrated the tendency of the circulating lymphocytes to decrease in number after animals had been exposed to the x-rays generated by gas tubes and because this decrease was, in many ways, similar to that observed in Experiments 1 to 4. Furthermore, there is a definite relation between the response of the various animals in a series, as determined by blood counts, to x-ray treatment.

TABLE IV.

Guinea Pig Receiving Approximately 3 Holzknecht Units.

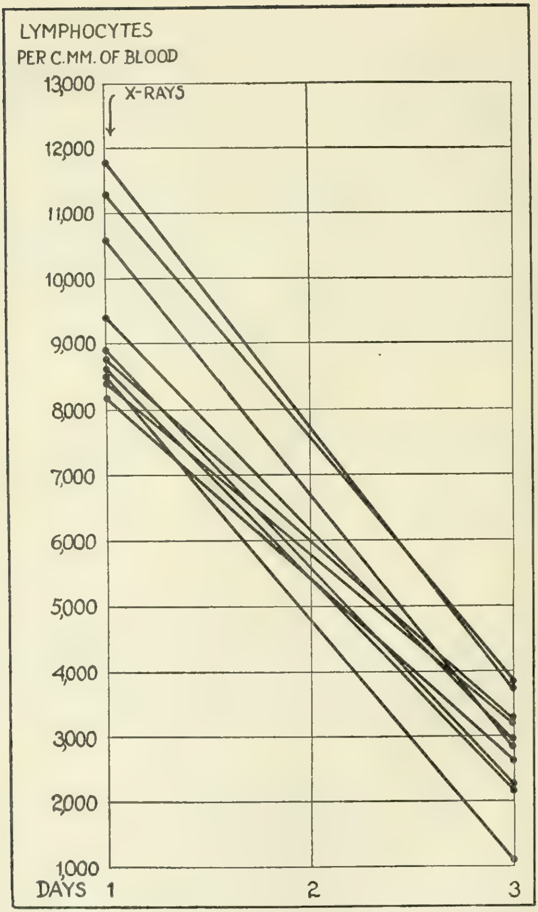
Day of experiment.	Length of time after x-rays.	Lymphocytes.		Polymorphonuclear cells.	
		Per cent.	Total No.	Per cent.	Total No.
	days				
1		36.7	10,606	61.7	17,831
5	2	16.3	4,283	81.3	21,138

Experiment 5.—Ten normal, adult, white rats were simultaneously exposed for the same length of time to unfiltered x-rays generated by a rather soft gas tube. Total white and differential blood counts were made on each animal immediately before and 48 hours after exposure. The effect of the x-rays on the circulating lymphocytes (total number per c. mm.) is shown in Text-fig. 3, where each line represents the difference, in a single rat, between the number of lymphocytes before and after treatment.

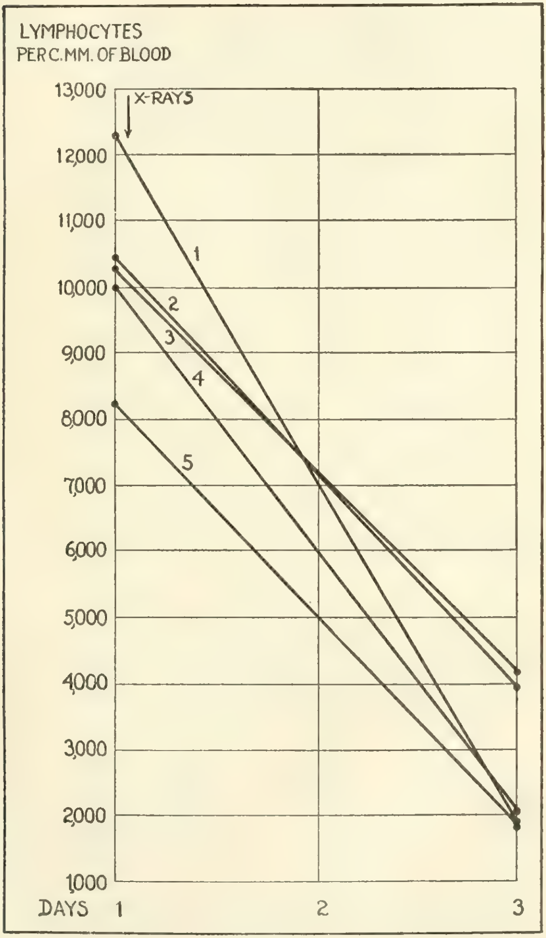
Experiment 6.—The procedure followed in Experiment 5 was repeated with five additional white mice and the results are shown in Text-fig. 4, in which the lines have the same significance as those in Text-fig. 3.

Experiment 7.—Four mice were simultaneously given seven treatments with unfiltered x-rays, the Coolidge tube being used. Blood counts were made on each animal before the first and 48 hours after the last x-ray exposure. The effect of the x-rays on the blood of each mouse is shown in the usual manner in Text-fig. 5; additional data of interest are given in Table V.[15] The factors at

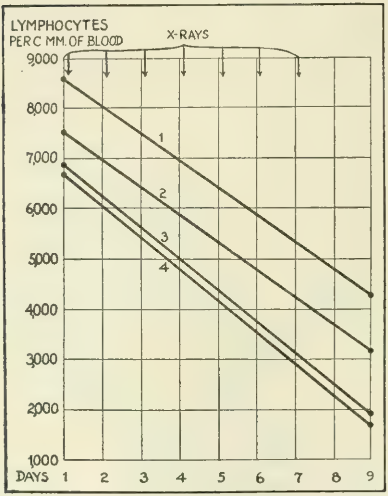
[15] The number of the animal in the curve and in the table corresponds, in this and the following experiments.



TEXT-FIG. 3. The effect of x-ray treatment on the circulating lymphocytes of ten white rats. In this and the following text-figures each line represents the difference in a single animal between the number of lymphocytes before and after treatment.



TEXT-FIG. 4. The effect of x-ray treatment on the circulating lymphocytes of five white mice.



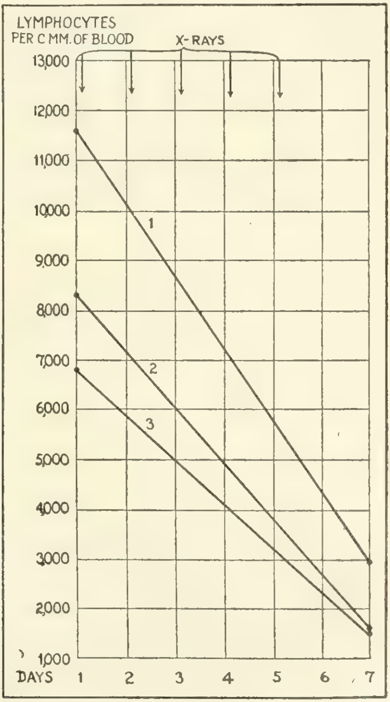
TEXT-FIG. 5. The effect of x-ray treatment on the circulating lymphocytes of four white mice.

TABLE V.

Four Mice Receiving 14 Holzknecht Units.

Mouse No.	Day of experiment.	Length of time after x-rays.	Lymphocytes.		Polymorphonuclear cells.	
			Per cent.	Total No.	Per cent.	Total No.
		days				
1	1		73.0	8,588	27.0	3,180
	9	2	55.3	4,301	44.7	3,477
2	1		90.3	7,527	9.6	800
	9	2	57.0	3,103	43.0	2,341
3	1		48.2	6,909	51.7	7,410
	9	2	42.0	1,914	58.0	2,643
4	1		51.0	6,687	49.0	6,424
	9	2	14.0	1,680	86.0	10,320

each exposure were: spark-gap 2¼ inches, milliamperes 10, distance from the target to the skin 12 inches, and time of exposure 2 minutes. This represents 2, at a single exposure, or a total of 14 Holzknecht units.



TEXT-FIG. 6. The effect of x-ray treatment on the circulating lymphocytes of three adult white rats.

Experiment 8.—Three adult white rats were exposed to the unfiltered x-rays generated by a Coolidge tube. The factors were: spark-gap 3 inches, milliamperes 10, distance from the target to the skin 12 inches, and time 4 minutes. This dose was repeated daily for 5 days. Each exposure represents 6, a total of

30 Holzknecht units being therefore given. White and differential blood counts made before the first and 48 hours after the last exposure are summarized in Table VI and total lymphocytes per c. mm. of blood at each determination charted in Text-fig. 6.

Experiment 9.—Three rabbits, after preliminary blood counts, were given seven daily, unfiltered doses of x-rays, each dose depending on the following factors: spark-gap 3 inches, milliamperes 10, distance from the target to the skin 12 inches, and time 4 minutes. This represents 6 at a single dose, or a total of 42 Holzknecht units. The dorsal and ventral surfaces of the animals were exposed to the rays on alternate days. Text-fig. 7 and Table VII refer to the blood counts on these animals.

Experiment 10.—Two rabbits were each given a single treatment with the unfiltered x-rays emitted by a Coolidge tube, which was controlled by the following factors: spark-gap 3 inches, milliamperes 20, distance from the target to the skin 12 inches, and time 4 minutes. This dose represents 11 Holzknecht units. Text-fig. 8 and Table VIII refer to the blood counts on these animals.

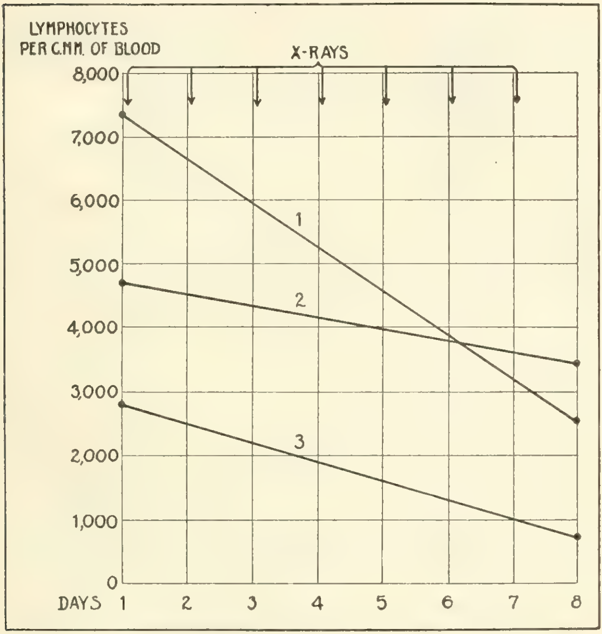
TABLE VI.

Three Rats Receiving 30 Holzknecht Units.

Rat No.	Day of experiment.	Length of time after x-rays.	Lymphocytes.		Polymorphonuclear cells.	
			Per cent.	Total No.	Per cent.	Total No.
		days				
1	1		89.7	11,683	10.3	1,342
	7	2	62.3	2,990	37.3	1,790
2	1		63.0	8,096	37.0	4,755
	7	2	16.3	1,593	83.7	8,182
3	1		79.2	6,831	20.7	1,785
	7	2	48.7	1,583	51.3	1,667

Experiment 11.—Two rabbits at a single exposure were treated with x-rays filtered through 3 mm. of aluminum, the dose approximating 5 Holzknecht units. This was determined by the following factors: spark-gap 9 inches, milliamperes 5, distance from the target to the skin 12 inches, and time 5 minutes and 20 seconds. Text-fig. 9 and Table IX refer to the circulating lymphocytes and polymorphonuclear leucocytes in these animals, before and 48 hours after exposure.

Experiment 12.—Three monkeys were each exposed to the unfiltered x-rays generated by a Coolidge tube for a total of seven treatments. Monkeys 1 and 2 had daily exposures, while Monkey 3 was given the seven treatments in 4 days. The factors at each treatment were: spark-gap 3 inches, milliamperes 10, distance from the target to the skin 12 inches, and time 4 minutes. At each exposure 6, or a total of 42 Holzknecht units were therefore given to each animal. Text-fig. 10 and Table X give the data for these monkeys.

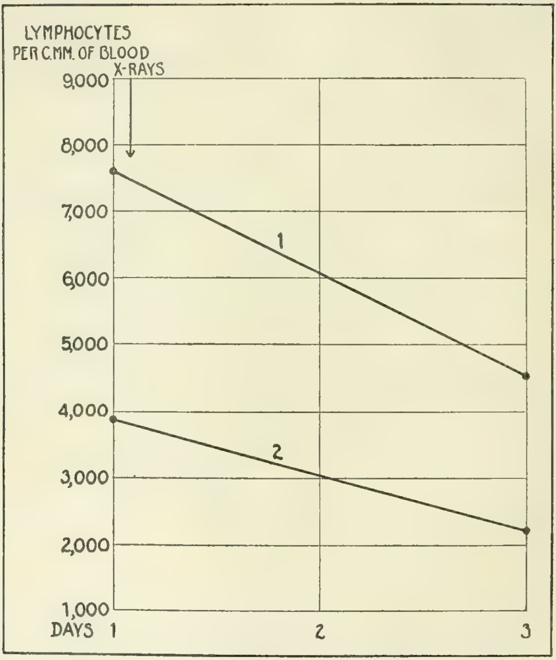


TEXT-FIG. 7. The effect of x-ray treatment on the circulating lymphocytes of three rabbits.

TABLE VII.

Three Rabbits Receiving 42 Holzknecht Units.

Rabbit No.	Day of experiment.	Length of time after x-rays.	Lymphocytes.		Polymorphonuclear cells.	
			Per cent.	Total No.	Per cent.	Total No.
		days				
1	1		55.3	7,383	42.0	5,607
	7	2	54.3	2,570	39.3	1,870
2	1		44.7	4,716	47.7	5,032
	7	2	39.7	3,462	55.7	4,857
3	1		29.7	2,866	64.0	6,176
	7	2	24.3	758	66.7	2,081

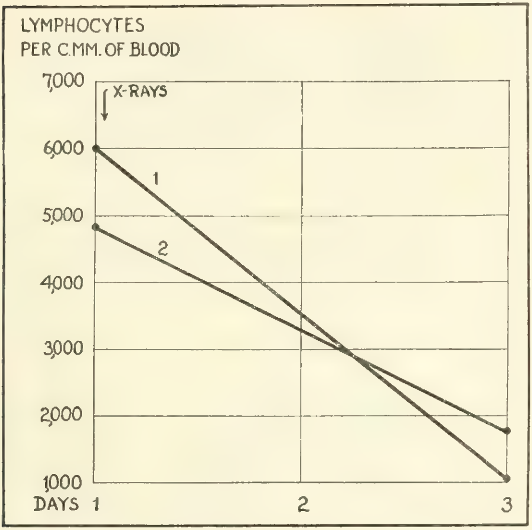


TEXT-FIG. 8. The effect of x-ray treatment on the circulating lymphocytes of two rabbits.

TABLE VIII.

Two Rabbits Receiving 11 Holzknecht Units.

Rabbit No.	Day of experiment	Length of time after x-rays.	Lymphocytes.		Polymorphonuclear cells.	
			Per cent.	Total No.	Per cent.	Total No.
		days				
1	1		66.3	7,591	31.3	3,584
	3	2	54.7	4,568	41.3	3,449
2	1		51.2	3,878	48.0	3,636
	3	2	32.3	2,196	65.6	4,461

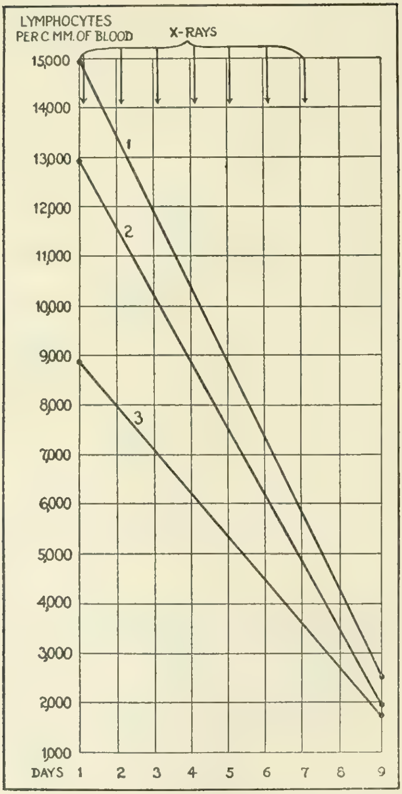


TEXT-FIG. 9. The effect of x-ray treatment on the circulating lymphocytes of two rabbits. The x-rays were filtered through 3 mm. of aluminum.

TABLE IX.

Two Rabbits Receiving Approximately 5 Holzknecht Units (Filtered).

Rabbit No.	Day of experiment.	Length of time after x-rays.	Lymphocytes.		Polymorphonuclear cells.	
			Per cent.	Total No.	Per cent.	Total No.
		days				
1	1		87.3	6,002	11.0	756
	3	2	44.7	1,017	50.7	1,153
2	1		54.0	4,860	39.7	3,573
	3	2	27.2	1,761	65.0	4,209



TEXT-FIG. 10. The effect of x-ray treatment on the circulating lymphocytes of three monkeys.

TABLE X.

Three Monkeys Receiving 42 Holzknecht Units.

Monkey No.	Day of experiment.	Length of time after x-rays.	Lymphocytes.		Polymorphonuclear cells.	
			Per cent.	Total No.	Per cent.	Total No.
		days				
1	1		59.7	14,925	34.3	8,575
	9	2	17.7	2,509	77.0	10,915
2	1		54.0	12,960	42.0	10,080
	9	2	15.0	1,980	77.5	10,230
3	1		54.7	8,861	43.7	7,079
	6*	22	17.7	1,744	81.3	8,008

* In this animal the seven treatments were given in 4 days.

DISCUSSION.

The immediate effect of the x-rays, in the dose employed in these experiments, is a sudden decrease in the circulating lymphocytes, evident in every curve and table in the series. The curves all represent total numbers of lymphocytes, small and large varieties combined, per c. mm. of blood. When the lymphocytes are studied in terms of percentage of total white blood cells the results are not so striking, and, while in most instances there is a definite fall in percentage as well as in actual numbers of these cells after x-ray treatment, an occasional instance is encountered where the percentile change is slight or absent. For example, whereas the total lymphocytes of Rabbit 1, Table VII, decreased from 7,383 before to 2,570 after exposure, the corresponding fall in percentage of lymphocytes was small; *i.e.*, from 55.3 to 54.3 per cent. The latter fall is well within the limits of counting error and is therefore negligible. It seems, therefore, that an estimation of the total number of lymphocytes per c. mm. of blood, determined by multiplying the total white count by the combined percentage of large and small lymphocytes, before and after x-ray treatment offers a more accurate indication of the effect on the blood than can be determined by following percentage figures only. This means, in other terms, that while the x-rays, in most instances, affect the lymphocytes selectively, occasional cases occur in which together with the lymphocytes the granular blood cells are also destroyed.

When the total number of circulating lymphocytes is plotted against the time in days a curve is formed which reaches its lowest level 48 hours after the administration of the x-rays (Text-figs. 1, *a*, *b*, and 2). Following this fall there is a primary rise, which reaches its acme from 3 to 5 days after the last x-ray exposure (3 days in Experiment 1, 9 days in Experiment 2, and 4 days in Experiment 3). A secondary fall then occurs, which reaches its lowest level from 5 to 12 days after treatment, and this is followed by a secondary and, as far as has been determined, permanent rise, which persists for at least 54 days after x-ray treatment (Text-fig. 2). The slight variation in the time relations of the various phases of the curves, shown in Text-figs. 1, *a*, *b*, and 2 are probably due to the fact that in Experiments 1 and 3 the entire dose of x-rays was given in a single day, while in Experiment 2 the seven doses were dispersed over an interval of 8 days. A comparison of Text-figs. 1, *a* and 2 shows that the primary rise occurred on the 3rd and on the 9th day respectively, while the secondary fall reached its lowest limit on the 5th and on the 21st day. It seems possible that the destructive action of the x-rays on the lymphocytes is first felt by these cells inthe general circulation and in the spleen (this corresponds to Warthin's view[5]), and that this accounts for the primary fall. In the meantime the lymphogenic cells of the other organs, lymph glands, bone marrow, etc., contribute cells to the blood, these being responsible for the secondary transient rise. When all the lymphogenic tissues have been affected by the x-rays the secondary fall in these cells is apparent in the blood, but it does not reach the extremely low level characteristic of the primary fall, because of beginning regeneration in the spleen. Regeneration of all the lymphogenic tissues later contributes to the permanent rise. It requires a considerable period of time before the lymphocyte-forming tissues regenerate entirely. In Experiment 2 for instance, the tissues are not contributing the normal numbers of lymphocytes to the circulation 54 days after the last x-ray treatment. The curve representing blood lymphocytes (Text-fig. 2) can be seen to be rapidly approaching the pre-treatment level at this time, however, and probably, had the observations been prolonged over a sufficiently long period of time, the lymphocytic elements would again have been present in the blood in normal numbers. When the x-ray treatments are distributed

over a number of days the issues are somewhat confused, and because of overlapping of phases the curves are not so sharply defined as when the entire dose is given in a single day. The primary fall in circulating lymphocytes, which in the case of the animals given x-rays in several doses is really the result of a series of primary falls, one following each treatment, is complete after 48 hours. However, the other phases, as may be seen from Text-fig. 2, are somewhat lengthened on this curve as contrasted with those given in Text-fig. 1, *a* and *b*.

From 24 to 48 hours after x-ray treatment there is, in most instances, a considerable increase in the polymorphonuclear neutrophilic leucocytes, which is well seen in the figures of Tables I and II. This is not evident in the rabbits followed (Tables VII, VIII, and IX) nor in the rats (Table VI). In mice (Table V) there is a tendency for these cells to exhibit a post-treatment rise, but this is not striking. The blood of the guinea pig showed a moderate increase in these cells after x-ray exposure.

When there is a primary rise in polymorphonuclear leucocytes, it is followed by a primary fall, which reaches its lowest level at a time when the lymphocytes are beginning to rise, the 6th day after x-ray exposure in Experiment 1, and the 3rd day in Experiment 2 (Tables I and II). Later there is a gradual rise to normal, about which level the number of neutrophilic cells fluctuates within wide limits. This is well seen in Table II. The polymorphonuclear cells are much less affected than the lymphocytes even when large doses of x-rays are given, and after the primary stimulation and later depression the return to the normal number per c. mm. occurs at a time when the lymphocytes are still at a very low level (Tables I and II). This seems to confirm Warthin's contention[5] that the bone marrow is affected somewhat later and to a more limited extent than the lymphogenic tissues proper. Circulating eosinophils and basophils, as well as large mononuclear and transitional leucocytes, usually share in the stimulations and depressions of the neutrophilic cells. This is what would be expected, as they all belong to the granular series and all originate in the bone marrow.

A glance at the curves shown in Text-figs. 3 to 10 leaves little to be said regarding the regularity with which the circulating lymphocytes of a series of animals of the same species respond to the same

dose of x-rays. Exceptions to the general rule of a quantitative relation between the reactions of the several animals of a series are uncommon. Apparently Rabbit 2 in Text-fig. 7 and Rabbits 1 and 2 in Text-fig. 9 belong in this category. The latter animals received the only treatment with a filtered dose of x-rays in the entire group reported, and it is not possible to draw conclusions from this single instance. The lymphocytic fall is evident, however, in both animals. Rabbit 2 in Text-fig. 7, then, is the only exception encountered and no explanation is apparent. There is a decrease in the circulating lymphocytes of this animal, following treatment with the x-rays, but this is not so great as in the companion rabbits, Nos. 1 and 3. Exceptions are naturally encountered in all biological experiments, and as this is the only striking instance found in the entire study it seems safe to disregard it and to accept the other results as manifesting accordance to a general law, inasmuch as they are regular and constant.

In Experiment 9 x-rays equivalent to 42 Holzknecht units destroyed, if all the rabbits are included, an average of 55.1 per cent, or, if Rabbit 2 is disregarded and the others (Nos. 1 and 3) with comparable lymphocyte curves are considered, an average of 69.4 per cent of the total number of circulating lymphocytes in the 48 hour interval following treatment. In the monkeys, given the same dose, Experiment 12, Table X, the effect of the x-rays was much more pronounced, 82.7 per cent of the circulating lymphocytes having disappeared 48 hours after treatment. It would seem that the monkey is more susceptible to the x-rays, in as far as the lymphocytic reaction represents an accurate measurement of the degree of susceptibility, than the rabbit. In the pony a dose of 160 Holzknecht units destroyed 95.4 per cent of the circulating lymphocytes, as determined 48 hours after treatment. In the monkey, Experiment 2, Text-fig. 2, Table II, 42 Holzknecht units were sufficient to destroy 93.1 per cent of the lymphocytes of the blood. This contrasts sharply with the three monkeys in Experiment 12, Text-fig. 10, Table X, in which the same dose destroyed an average of 82.7 per cent, and in the animal most affected, No. 3, but 84.7 per cent.

The lymphogenic tissues of the cat seem to be more susceptible than those of any other animal studied, inasmuch as 3 Holzknecht

units were sufficient to destroy 89.2 per cent of the circulating lymphocytes. In some of the experiments it was not possible to determine accurately the doses of x-rays given, but from the information at hand it seems that different species of animals vary considerably in their response to a given dose of x-rays. Those studied here would seem to follow a series, progressing from the most to the least susceptible, somewhat as follows: cat, monkey, guinea pig, rabbit, rat, mouse, and pony.

SUMMARY.

1. X-rays in large doses affect the lymphocytes before any of the other circulating cells.

2. There is a sharp fall in the total number of circulating lymphocytes, which is complete 48 hours after x-ray treatment.

3. Following the immediate decrease in the circulating lymphocytes there is a primary rise, followed by another fall, which in turn is followed by a permanent rise of these cells to normal.

4. The effect of the x-rays on different species of animals varies considerably, but in those studied, cat, monkey, guinea pig, rabbit, rat, mouse, and pony, the selective action on the lymphocytes was in all instances apparent.

5. When several animals of the same species are given the same dose of x-rays, the effect on the circulating lymphocytes seems to be quantitatively parallel, when determined by blood counts.

6. The polymorphonuclear neutrophilic leucocytes, when affected at all, increase in number immediately after the administration of the x-rays and then tend to decrease below their normal level. This decrease is followed by a return to normal many days before the lymphocytes reach their original level.

7. The other cells of the blood follow the neutrophilic curve.

8. Percentage figures, as determined by differential blood counts, do not give an accurate indication of the effect of the x-rays. It is only when these are multiplied by the total white blood count that a figure, representing the total number of cells of the series per c. mm. of blood, is obtained, which varies to the stimulus in a constant manner, the variations being practically quantitative.

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STUDIES ON X-RAY EFFECTS.

II. STIMULATIVE ACTION ON THE LYMPHOCYTES.*

BY MARGUERITE M. THOMAS, HERBERT D. TAYLOR, M.D., AND WILLIAM D. WITHERBEE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 27, 1918.)

Taylor, Witherbee, and Murphy[1] have reported on the destructive action of x-rays on the circulating lymphocytes, confirming and extending the earlier work on this subject. It was noted by Murphy in his studies on x-ray effects[2] that while large doses destroyed, a small dose of x-rays would bring about a stimulation of the lymphocytes. This observation was later applied experimentally.[3] In the earlier experiments the older type of x-ray tube was used, and it was practically impossible to establish a standard and uniform dose. With the introduction of the Coolidge tube the difficulty was eliminated to a large extent, and there was an opportunity to check this observation and extend it.

Mice have not been used here as in the previous experiments for the reason that blood counts could not be made on these animals more frequently than once a week without causing too marked a fluctuation.

EXPERIMENTAL.

Brown rabbits of the same relative size were used in the nine experiments. All the animals were kept in separate cages. Several blood counts were made on these normal rabbits, and they were then exposed to the rays of a Coolidge tube. A dose of low penetration

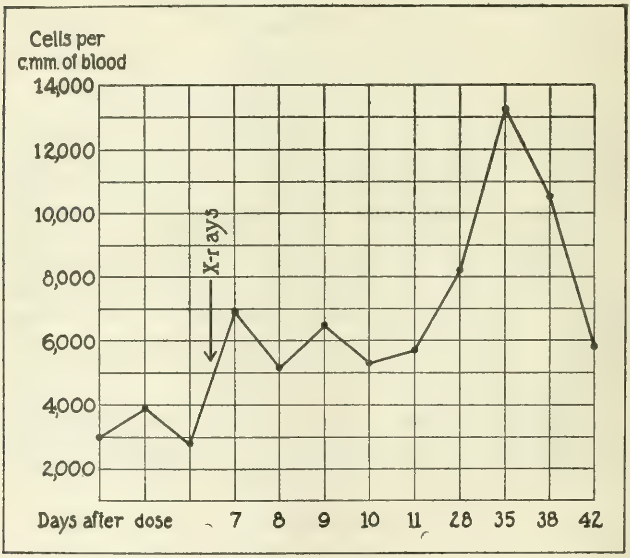
*This investigation was carried out by means of funds from the Rutherford Donation.

[1] Taylor, H. D., Witherbee, W. D., and Murphy, Jas. B., *J. Exp. Med.*, 1919, xxix, 53.

[2] Murphy, Jas. B., unpublished observation.

[3] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.

was applied to the dorsal area: the spark-gap measured $\frac{7}{8}$ inch, the milliamperage was 25, the distance from the target to the back 8 inches, and the time of exposure 20 minutes. The temperature 8 inches from the target was 31°C. In almost every case a blood count made 48 hours after exposure showed a slight drop in the lymphocytes.

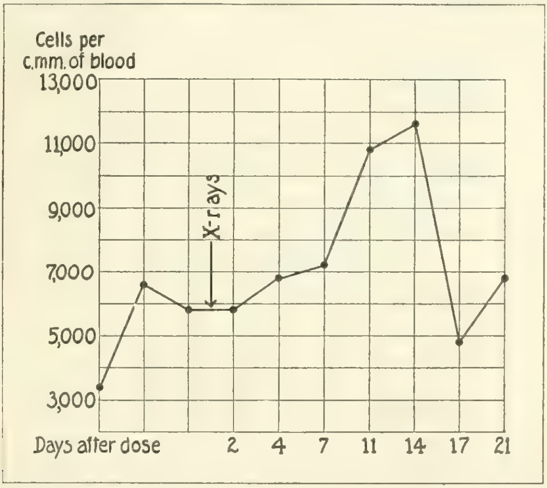


TEXT-FIG. 1. Effect of unfiltered x-rays of low penetration on the lymphocytes of a rabbit.

Experiment 1 (*Text-Fig. 1*).—Three consecutive counts were made, the lymphocytes numbering 3,000, 4,000, and 2,800 respectively, and the rabbit was then exposed to x-rays. The count made 1 week later showed a stimulation in the cells, the number of lymphocytes being 6,900. Counts made at intervals showed slight fluctuation until the 28th day, when the count reached 8,300. On the 35th day there were 13,300 absolute lymphocytes. In the course of the 7 days following the last count the number of cells fell to 5,800.

Experiment 2 (*Text-Fig. 2*).—Considerable fluctuation was shown in the normal counts. The first count 48 hours after exposure to x-rays showed little or no change, and for 7 days afterwards there was little increase, the lymphocytes numbering 5,000, 6,000, and 7,000 respectively, but on the 11th day the number had risen to 10,500, and on the 14th day to 11,600. They fell to 4,800 on the 17th day and in the last count rose to 6,900.

Experiment 3.—The standardizing counts were 6,900, 6,370, and 7,000. 48 hours after exposure to the x-rays a slight fall was noted, the number of cells



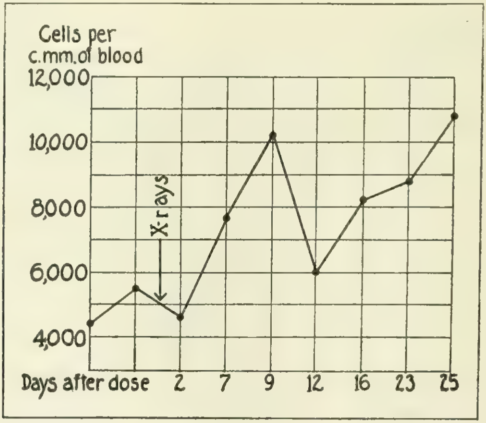
TEXT-FIG. 2. Effect of unfiltered x-rays of low penetration on the lymphocytes of a rabbit.

at that time being 5,100. On the 4th day there were 5,300 lymphocytes, and 6 days afterwards 10,240, showing marked stimulation. On the 8th day the number had decreased to 6,100 and on the 15th day to 4,000. After that there was a slight rise (6,500) on the 18th day and also on the 23rd, followed by another fall to 3,410. A rise to 8,200 was noted on the 64th day, and the last count, at which the number of cells was 6,300, was made on the 70th day after exposure.

Experiment 4.—4,300, 4,670, and 4,900 were the lymphocyte counts of a rabbit before x-ray treatment. 48 hours after exposure there was a slight increase (6,600). The 4th day the number of cells was 4,500, the 6th day 6,500, and the

15th day, when the last count was made, 5,979. This rabbit did not show a stimulation as did those of the preceding experiments.

Experiment 5 (*Text-Fig. 3*).—At the two counts preceding x-ray treatment there were 4,560 and 5,300 lymphocytes respectively. 48 hours after exposure to the x-rays there was a slight fall to 4,600, and on the 7th day a rise (7,600) which continued to the 9th day and at that time reached 10,200. On the 12th day there was a slight decrease, but from that time to the 25th day, on which the last count was made, there was a continued rise in the number of lymphocytes, which finally reached 10,900.



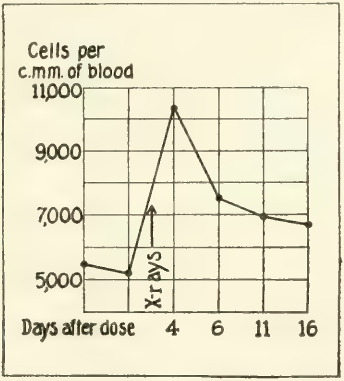
TEXT-FIG. 3. Effect of unfiltered x-rays of low penetration on the lymphocytes of a rabbit.

Experiment 6.—The normal counts showed a great fluctuation, the lymphocytes numbering 3,500 at the first count, at the second 6,100, and at the third 6,290. 48 hours after x-ray exposure they fell to 3,600, rose on the 4th day to 6,400, and fell again on the 6th day to 3,800. The cell counts, which were made at intervals until the 25th day after exposure, continued to rise and fall, never reaching a point beyond 5,900 or below 3,000.

Experiment 7.—The normal counts in this instance were 4,900 and 5,800. 48 hours after exposure to the x-rays the cells numbered 5,600, the 4th day 5,400, the 6th 5,700, and the 8th 6,180, showing a slight increase. On the 13th day the number of cells was 5,600, and on the 20th day, when the last count was made, 5,750.

Experiment 8.—2,270, 5,340, and 2,267 were the three consecutive lymphocyte counts made before x-ray treatment on a rabbit. 48 hours after treatment there was little or no change, the cells numbering 2,200. On the 4th day there was a rise to 4,800, on the 6th to 7,900. 11 days after exposure the number was 6,560. Subsequently there was a decline to 3,900 (13th day) and finally, on the 25th day, to 3,000.

Experiment 9 (*Text-Fig. 4*).—Two normal counts, the first 5,500, the second 5,100, were followed by the short x-ray dose. The 48 hour count was not made on this rabbit. The first count following exposure was made 4 days afterwards, the lymphocytes then numbering 10,390. On the 6th day there was a fall to 7,500, on the 11th day to 6,900, and on the 16th day to 6,700.

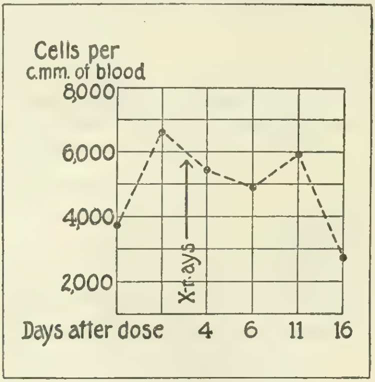


TEXT-FIG. 4. Effect of unfiltered x-rays of low penetration on the lymphocytes of a rabbit.

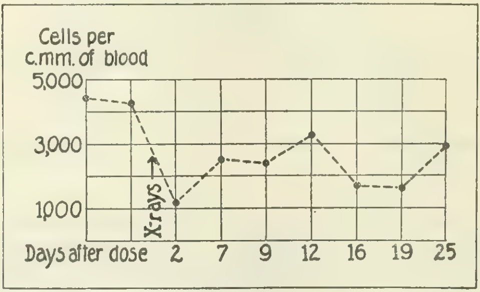
A comparable dose of filtered x-rays[4] was used also on a smaller number of brown rabbits (spark-gap 6 inches, milliamperage 5, distance from the target to the back 10 inches, time 26 minutes and 57 seconds). The rays were filtered through 3 mm. of aluminum. The animals were exposed in the same way (over the dorsal area) and kept under the same conditions as those of the preceding experiments.

[4] Remer, J., and Witherbee, W. D., *Am. J. Roentgen.*, 1917, iv, 303.

Experiment 1, a (*Text-Fig. 5*).—In the two counts preceding x-ray exposure the number of lymphocytes was not very constant, being 3,700 at the first and 6,600 at the second. After the filtered x-ray dose (6 inch spark-gap, milliamper-



TEXT-FIG. 5. Effect of filtered x-rays on the lymphocytes of a rabbit.



TEXT-FIG. 6. Effect of filtered x-rays on the lymphocytes of a rabbit.

age 5, distance from the target to the back 10 inches, and time 26 minutes and 57 seconds) the count showed little change, 5,470 in the first count, which was made on the 4th day, 4,900 on the 6th day, 5,900 on the 11th day, and 2,733 on the 16th day.

Experiment 2, a (Text-Fig. 6).—The lymphocytes numbered 4,400 and 4,350 at the two counts preceding x-ray treatment. 48 hours after exposure there was a marked fall (1,230), and on the 7th day a slight rise (2,500). On the 9th day the count was similar to that of the 7th; on the 12th it was 3,500, on the 16th 1,700, on the 19th 1,720, and on the 25th 2,981. There is no sign of stimulation in Experiment 2, *a*.

DISCUSSION.

It is of interest to note in these experiments that the x-ray dose used was of low penetration, the spark-gap being under an inch. The use of a larger spark-gap with apparently the same dose o x-rays did not give a stimulation. This suggests that the effect o the lymphoid organs is not the result of a direct action of the rays but is secondary to changes brought about either in the circulating blood or in the superficial tissues. The amount of x-rays penetrating to the deeper structures with this dose must be infinitesimal.

Another question arises as to the nature of the energy generated by the x-ray tube operated on so small a spark-gap. This point has not yet been taken up, but it is conceivable that other factors than the pure x-rays may play a part.

The results obtained in this small series of animals would not in themselves be accepted as conclusive evidence but are of interest principally as a parallel to our histological studies.[5] It is conceivable that a marked stimulation may be taking place in the lymphoid organs without a proportionate number of these cells being thrown into the circulation. The question in itself offers an interesting problem of just what determines the number of cells in the circulation. It is well known that individuals with normal counts react differently in the number of cells thrown into the circulation in response to infections. So here, even with marked stimulation taking place in the lymphoid tissue of the glands and spleen, in only a part of the animals perhaps could we expect this stimulation to be evidenced by an increase in the number of lymphocytes in the circulating blood.

[5] Nakahara, W., *J. Exp. Med.*, 1919, xxix, 83.

SUMMARY.

This study consists of blood counts on nine rabbits after an exposure to x-rays of a $\frac{7}{8}$ inch spark-gap, milliamperage 25, distance from the target 8 inches, and time of exposure 20 minutes.

In seven of the nine animals there resulted an increase of the circulating lymphocytes. In five of these the increase was marked and in two others definite but not striking.

Of the two animals which showed no stimulation one showed marked fluctuation of counts both before and after x-rays and the other little or no change.

The higher penetrating dose (6 inch spark-gap, milliamperage 5, distance from the target 10 inches, time 26 minutes and 57 seconds) given to two animals produced no appreciable stimulation.

[Reprinted from The Journal of Experimental Medicine, January 1, 1919, Vol. xxix, No. 1, pp. 83–87.]

STUDIES ON X-RAY EFFECTS.

III. Changes in the Lymphoid Organs after Small Doses of X-rays.

By WARO NAKAHARA, Ph.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 8 and 9.

(Received for publication, October 21, 1918.)

The observations of previous investigators have emphasized the fact that x-rays are preeminently and selectively destructive to lymphoid cells (Heineke[1]). Thomas, Taylor, and Witherbee[2] have shown that small doses of x-rays will bring about a stimulation of the circulating lymphocytes, confirming the earlier observations made in this laboratory.[3] As in the experiments on the effect of heat on the circulating lymphocytes,[4] there was a fall immediately after exposure, followed by a more or less gradual rise.

The histologic findings in the lymphoid tissues of mice treated with heat have already been reported,[5] and it has been shown that the lymphocytosis induced by heat is due to the proliferation of certain cells in reaction to the extensive destruction of the tissue by this agent. *A priori*, since x-rays are known to be destructive to lymphoid tissue, the lymphocytic changes observed seemed to be due to a similar cause. The experiments to be reported here were undertaken in order to determine this point.

[1] Heineke, H., *Münch. med. Woch.*, 1903, l, 2258; *ibid.*, 1904, li, 1382; *Mitteil. Grenzgeb. Med. u. Chir.*, 1905, xiv, 21.

[2] Thomas, M. M., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 75.

[3] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.

[4] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 1.

[5] Nakahara, W., *J. Exp. Med.*, 1919, xxix, 17.

EXPERIMENTAL.

Six normal rabbits of the same color (white), and of approximately the same age, were treated with the same dose of x-rays as was used by Thomas, Taylor, and Witherbee (spark-gap $\frac{7}{8}$ inch, milliamperage 25, distance 8 inches, time 20 minutes). The animals were killed at intervals, and tissues were taken (*a*) immediately, (*b*) 48 hours, (*c*) 4 days, (*d*) 7 days, (*e*) 10 days, and (*f*) 14 days after treatment. This experiment has been duplicated with six brown rabbits. In the second experiment, however, animals were killed two by two (*a*) 24 hours, (*b*) 3 days, and (*c*) 14 days after they were x-rayed, these periods being found to be critical from the result of the first experiment.

For fixation, Carnoy's chloroform-alcohol-acetic (6:3:1) was easy to use and gave good results. Its use saved much time by dispensing with the necessity of passage through the grades of alcohol. Some tissues were fixed in Zenker's fluid for control. The staining was done almost exclusively with Heidenhain's iron-hematoxylin, though a few sections were stained by the ordinary method of hematoxylin and eosin.

Results.

Spleen.—The general histologic condition of the spleen immediately to 24 hours after the treatment is approximately normal. Necrotic cells are extremely scarce in both the nodule and the pulp. The number of mitotic figures in the germinal center of the nodule varies; as a rule, it shows in section a few, sometimes several, and more rarely ten. In the normal spleen of the adult rabbit the germinal centers contain but few mitotic figures, and frequently none. The excessive abundance of mitotic figures after treatment may therefore be taken to indicate the stimulation of these cells.

Sections taken 48 hours after treatment show a condition apparently identical to that just described. The number of necrotic cells in the pulp seems to be slightly larger if anything, but not large enough to be regarded as abnormal.

4 days after the treatment the signs of stimulation become most marked. While the general histologic condition remains unchanged,

the number of mitotic figures in the germinal center of the nodule is decidedly increased. The germinal center is seen in the section to contain usually several, but sometimes about ten, mitotic figures (Figs. 1 and 2). A comparison of this with the normal condition, in which the proliferative activity in the germinal center is limited, seems to warrant the conclusion that the small dose of x-rays acts as a stimulant to the proliferation of the lymphoid cells.

Sections were taken on the 7th, 10th, and 14th days after treatment. The general histologic condition of all the sections was found to be normal, with the exception of a slightly enhanced mitosis. In the sections taken on the 7th day the number of mitotic figures in the germinal center of the nodule was seen to be smaller, and the tendency to decrease was manifested even more markedly in the sections taken later (on the 10th or 14th day). Even in the later sections, however, the proliferative activity of the cells, judged from the standpoint of mitosis, seemed slightly above the normal.

Lymph Glands.—In addition to the mesenteric lymph gland, cervical, axillary, and inguinal glands were taken for comparison. The changes in these glands were found to be so nearly uniform in character that a general description will apply to all of them.

The most striking deviation from the normal in the histology of lymph glands immediately after the treatment is the great abundance of mitotic figures in the nodule. The dividing cells are not localized, but are found all over the nodule. Some mitotic figures are seen in the internodular spaces in the cortex and also in the medulla, where, as a rule, very few if any division figures are normally present. The whole tissue was seen to be almost entirely free from degenerating cells. Some evidences of necrosis were, of course, observed here and there, but they were no more conspicuous than those seen in normal tissue.

48 hours after treatment the signs of stimulation of the cells were even more evident than immediately after treatment. All nodules contained excessively large numbers of dividing cells, each of the ordinary sized nodules showing in a section at least ten, and not infrequently as many as twenty mitotic figures (Figs. 3 and 4). The general condition of the gland is similar to that immediately after the treatment, except that there is a slight increase of necrotic cells, mainly in the pulp spaces.

Mitotic figures appear slightly less numerous in the tissue taken 4 days after treatment. The nodule usually shows several mitotic figures in a section. These conditions may still be taken as slightly abnormal. The occurrence of the dividing cells is more or less localized in the nodule, and in many instances they constitute rather typical germinal centers.

In sections taken 7, 10, and 14 days after the treatment, conditions are similar to those just described. The slightly excessive occurrence of mitosis as late as the 14th day is well shown in many nodules in the form of actively proliferating cells of the germinal center.

DISCUSSION.

As far as the results of lymphocyte counts show, the nature of lymphocytosis induced by heat and of that brought about by x-rays is indistinguishable, since the lymphocytic changes are exactly parallel in both cases; *i.e.*, always with a characteristic fall preceding the marked rise. The idea that the phenomenon of lymphocytosis is of the same nature, regardless of whether the agent used for its production is heat or x-rays, seems probable, especially when we recall the results of Heineke[1] and Warthin,[6] who have shown that the effect of x-rays on lymphoid tissue is, in the main, similar to that of heat, as described by us in another paper.[5] They used the x-rays, however, in different dosage from ours, and did not, furthermore, make observations during the critical period after the x-ray treatment, when an excessive multiplication of the cells may possibly take place. Notwithstanding the apparent similarity in the nature of the two phenomena which have been pointed out, the results of the experiments described in the present paper show conclusively that the lymphocytosis induced by the small dose of x-rays is due to the primary stimulative effect of the agent and hence is fundamentally different in nature from the similar lymphocytic change induced by heat, which is a sort of regenerative phenomenon.

Throughout the course of the experiment no indication has been observed that suggests the injurious effect of the dose upon any of the

[6] Warthin, A. S., *Physician and Surg.*, 1907, xxix, 1.

lymphoid tissues examined. On the other hand, mitotic figures were seen to become gradually more abundant after the treatment. In the spleen this enhanced proliferative activity of the cell reached its height about 4 days after the treatment, and the more or less distinct indications of the stimulation persisted in the germinal center up to the 14th day after the treatment. In the lymph glands the stimulative change is distinctly manifested earlier and is more extensive than in the spleen.

These histologic findings are in harmony with the results of the blood cell counts, which show that the increase of lymphocytes becomes most pronounced about 1 week after the treatment. If the lymphocytosis is due to the stimulation of the lymphoid tissues, the latter should show the change before the former becomes evident, and this is apparently what takes place.

SUMMARY AND CONCLUSION.

1. The small dose of x-rays applied to the rabbit has no appreciable destructive effect on the lymphoid tissue.

2. Indications of stimulation of the lymphoid tissue appear immediately after the treatment, become most pronounced in 2 (in lymph glands) to 4 (in the spleen) days, and persist, in a slight degree, up to the 14th day.

3. These facts suggest that the lymphocytosis induced by the small dose of x-rays is due to a primary stimulative effect upon the lymphoid tissue of the animal.

EXPLANATION OF PLATES.

Plate 8.

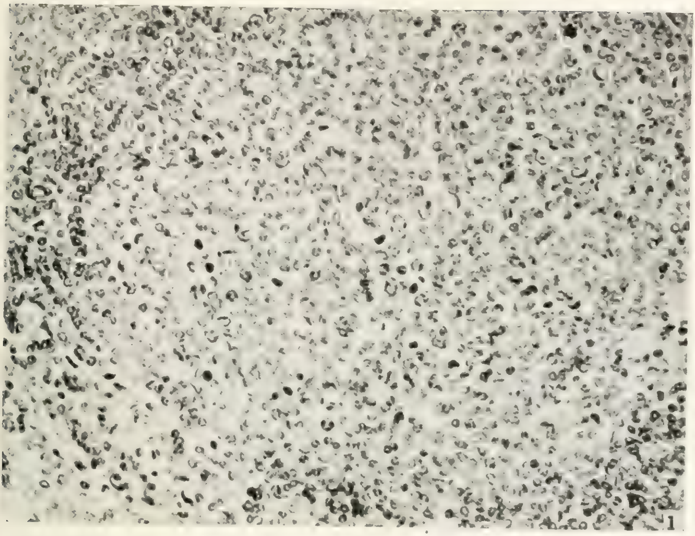
Fig. 1. A splenic nodule, 4 days after the treatment, showing extensive stimulation. × 350.

Fig. 2. The same, showing mitotic figures (*M*) in higher magnification. × 1,000.

Plate 9.

Fig. 3. A nodule of the mesenteric lymph gland, 48 hours after the treatment, showing intense stimulation. × 350.

Fig. 4. The same, showing mitotic figures (*M*) in higher magnification. × 1,000.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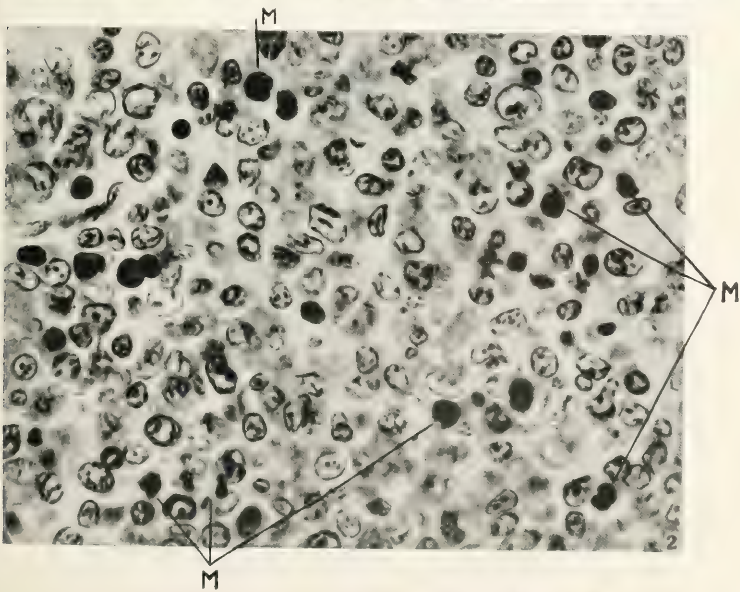
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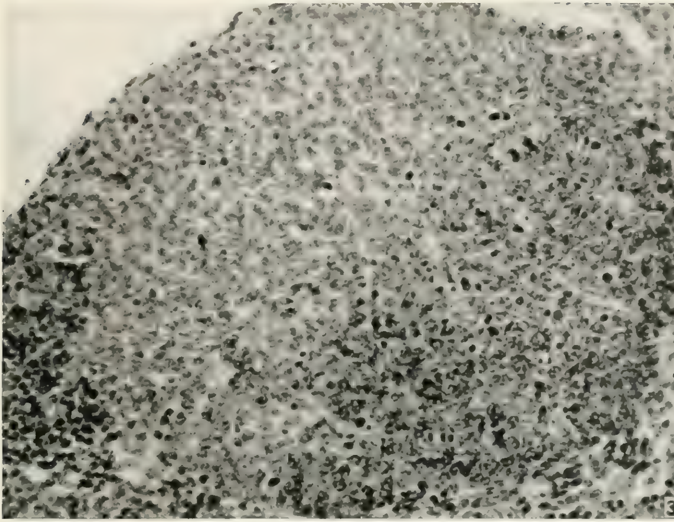

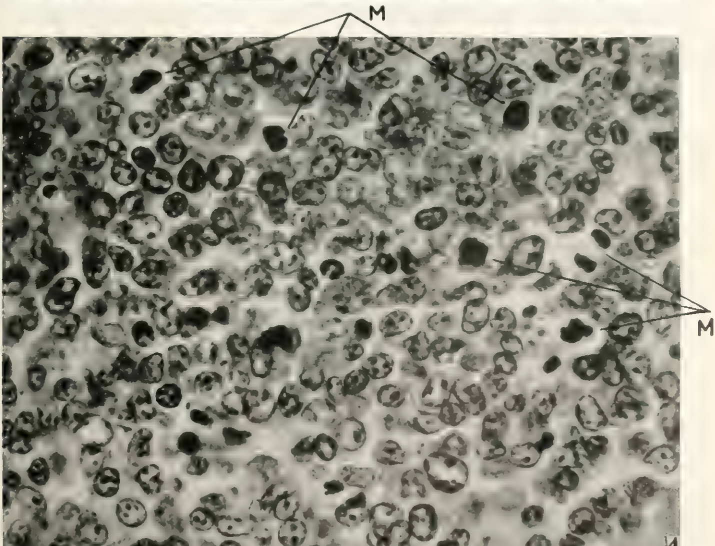

(Nakahara: Studies on x-ray effects. III.)

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STUDIES ON X-RAY EFFECTS.

IV. DIRECT ACTION OF X-RAYS ON TRANSPLANTABLE CANCERS OF MICE.*

BY ELSA HILL, JOHN J. MORTON, M.D., AND WILLIAM D. WITHERBEE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 21, 1918.)

It has been shown in this laboratory that x-rays administered in doses sufficient to destroy a large proportion of the lymphoid tissue reduce the resistance of the animal to transplanted cancer.[1] Furthermore, both potential and established induced immunity can be destroyed by a similar process.[2] It has also been shown that small doses of x-rays sufficient to stimulate the lymphocytes increase the resistance to cancer.[3] These observations bring up a number of interesting points with regard to x-rays as a therapeutic agent in the treatment of cancer. The literature on this subject is so extensive and contradictory that no attempt will be made to review the previous work. The question of immediate interest to us in this investigation is whether or not x-rays, even in a dose above that possible for therapeutic purposes, will kill the cancer cell. It is necessary in the light of the experiments mentioned above to rule out the action of this agent on the animal itself.

To determine the cumulative, as well as the immediate effect of the direct action of powerful doses of x-rays upon tumor cells, we undertook to grow a Bashford mouse tumor (Adenocarcinoma No. 63) in successive generations of white mice, subjecting the tumor to like doses of x-rays after excision and before inoculation at each transplantation. An actively growing tumor which had been propagated

* This investigation was carried out by means of funds from the Rutherford Donation.

[1] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

[2] Murphy, Jas. B., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 1.

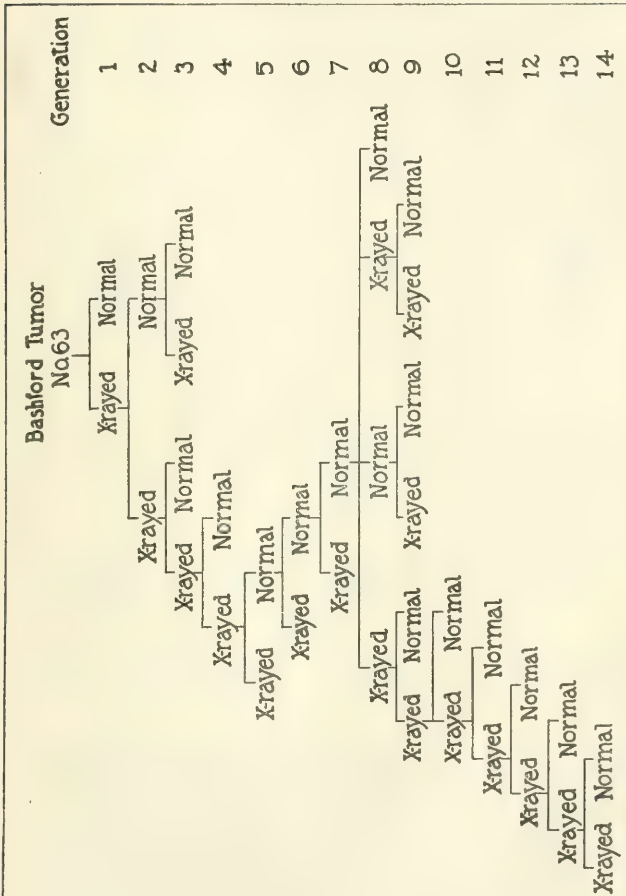
[3] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 800.

in the laboratory for some time was used, and an x-ray dose selected which was purposely above the range of therapeutic dosage: Coolidge tube, spark-gap 8 inches, milliamperes 5, distance 6 inches, and time 2 minutes and 35 seconds. The percentage of takes and rate of growth of the tumor were observed in fourteen generations, extending over a period of 17 months with eleven exposures between transplantations.

Later, considering the possibility of greater absorption of less penetrating rays, we undertook a similar experiment with the following dose: Coolidge tube, spark-gap 1 inch, milliamperes 25, distance 8 inches, and time 20 minutes. In this experiment the tumor was observed in four generations.

Method.

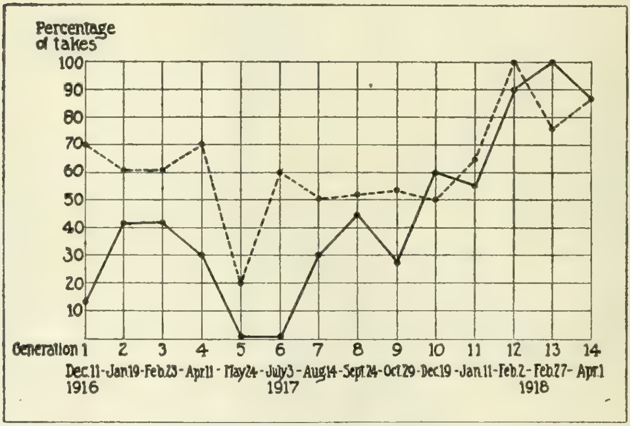
In both experiments (Nos. 1 and 2), the technique used was as follows: Wherever possible a healthy, actively growing tumor, approximately 1.5 by 1.5 cm., was chosen for inoculation. The mouse was killed and the tumor removed under aseptic conditions. It was then cut into halves and each half placed in a small Petri dish, covered with a single layer of sterile gauze, and set into a large Petri dish containing sufficient salt solution to keep the gauze slightly moist. One-half remained in the laboratory at room temperature, while the other half was subjected to x-rays. The temperature at the surface of the gauze did not rise above 33°C. in Experiment 1, or 42°C. in Experiment 2. The x-rayed portion of the tumor was then divided into small pieces of uniform size. Care was taken to select for inoculation the outer actively growing portions of the tumor. Asepsis was observed throughout the experiment. Single pieces of the tumor were loaded into hollow needles and ten normal white mice (young adults) were inoculated in the right groin. In loading the needles care was taken to macerate the tissue as little as possible. The control half of the tumor which received no x-rays was then inoculated into ten mice in exactly the same manner. In Experiment 1 the x-rayed half remained out of the body 45 to 50 minutes and the control half 1 hour. In Experiment 2 the x-rayed half remained out of the body 60 to 70 minutes and the control half $1\frac{1}{4}$ hours. The tumors resulting from the inoculations were measured at periods of 1 week



TEXT-FIG. 1. The generations of tumor transplantations for Series 1, extending over a period of 17 months. The final tumor transplant had been exposed to eleven treatments of x-rays during this period.

until the death of the mice. A healthy tumor was selected from the x-rayed series unless because of an epidemic or some other cause this was impossible, when one of the control tumors was chosen for treatment and an inoculation was made into another series of twenty mice in the manner described.

The first series of transplants was begun December 11, 1916, and terminated May 9, 1918 (Text-fig. 1). In Generations 5 and 6 the

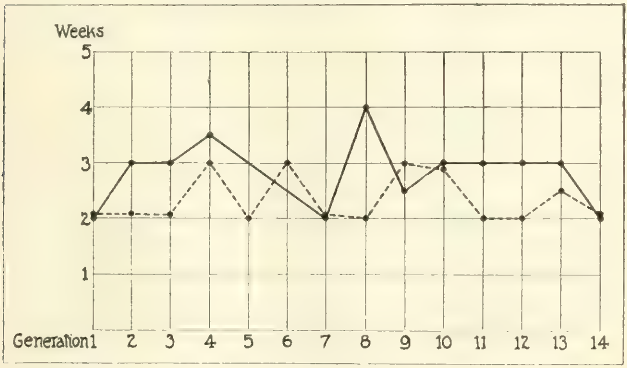


Text-Fig. 2. A comparison of the number of takes between x-rayed and untreated cancer on inoculation into mice. ....Controls. ———X-rayed cancers.

percentage of takes is inaccurate, probably much too low, on account of an epidemic among the mice which occurred before the usual time of appearance of tumors. For the transplants at this period, on account of the loss from the epidemic, the tumors of the next generation were taken from the control series. If we disregard the sudden drop in those two generations, the percentage of takes among the mice receiving x-rayed portions of the tumor remained between 25 and 45 during the first year, or nine generations. At the same time the percentage of takes among the control mice was between 50 and 70.

From the tenth generation to the fourteenth the percentage of takes does not remain consistently higher among controls and in both x-rayed and normal groups the percentage rises even to 100 (Text-fig. 2).

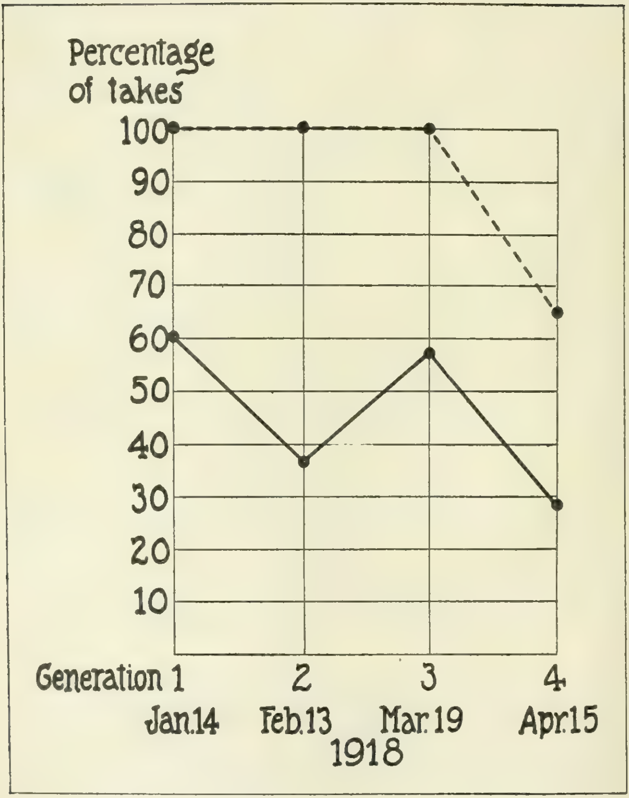
The rate of growth of the tumors was judged from curves representing the average time of appearance of the tumors and the average size 5 weeks after transplanting. As these two curves were essentially similar, only the former is illustrated (Text-fig. 3). In less than half the generation groups the tumors appeared a week earlier in the control mice than in those which had received the x-rayed



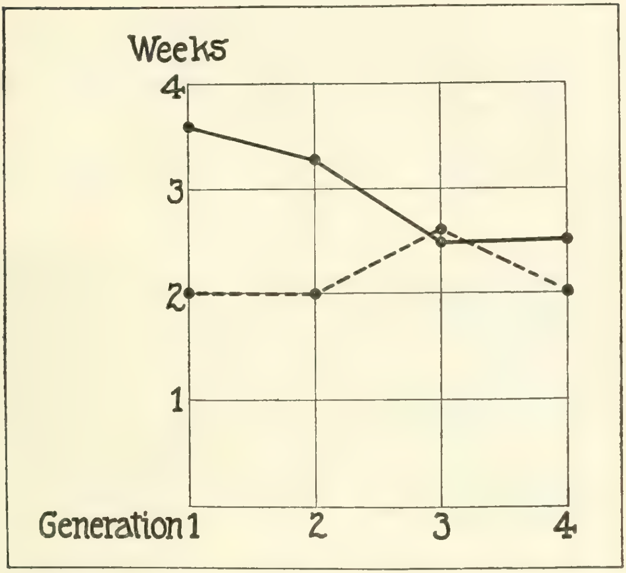
TEXT-FIG. 3. A comparison of the average time of appearance of tumors with and without exposure to x-rays. ....Controls. ————X-rayed cancers.

tumor. The difference in size between the tumors of the two groups 5 weeks after transplantation is slight and not consistent.

Experiment 2 was begun January 14, 1918, and terminated May 9, 1918. Four successive generations were observed. Without a much longer series of transplants it is impossible to judge the cumulative effect of this dose of x-rays on the general virulence of the tumor. The difference in the percentage of takes is consistently much more marked in this series than at any point in the first series (Text-fig. 4). Initially the rate of growth of the tumors receiving x-rays was also greatly retarded (Text-fig. 5).



TEXT-FIG. 4. A comparison of the percentage of takes for Series 2. ....Controls. ————X-rayed cancers.



TEXT-FIG. 5. Average time of appearance of tumors, x-rayed and untreated, in Series 2. ....Controls ———X-rayed cancers.

DISCUSSION.

The present tendency of workers on x-ray therapy of cancer is to devise methods of increasing the amount of x-rays delivered at the location of the cancer process. In the light of our observations there is one point which should be taken into consideration; that is, whether or not we are justified in using a procedure which apparently only inhibits the cancer temporarily, while it incidentally lowers the resistance of the individual to the growth. It is well recognized that a proportion of cancers held in check for a time by x-ray treatment will later grow more rapidly. It is not possible to form an idea of what proportion of the total number of patients treated show this result, as few completely and accurately controlled series have been

published. Blood counts on a number of these individuals have been made in this laboratory, and they all showed remarkably low lymphoid counts. Our work, however, has been done mainly on cancer of mice, and we are therefore not warranted in drawing sweeping conclusions until there is more careful confirmation from studies on man. We feel justified, however, in suggesting that powerful doses of x-rays which are only capable of inhibiting cancer growth for a time may bring about eventually a lowered resistance to a return of the disease process.

CONCLUSIONS.

These experiments indicate that the direct action of x-rays in more powerful doses than can be applied therapeutically is somewhat injurious to tumor cells, but by no means destroys them. Experiment 1 also indicates that the cancer cells establish a resistance to the x-rays after repeated doses. This harmonizes with the experience of clinicians who have succeeded in checking cancerous growths for some time but reach a point where no response can be effected by repeated doses. The rays of low penetration used in Experiment 2 are apparently more harmful to tumor cells than the penetrating rays used in Experiment 1.

[Reprinted from The Journal of Experimental Medicine, January 1, 1919, Vol. xxix, No. 1, pp. 97-114.]

BLOOD COUNTS IN EXPERIMENTAL POLIOMYELITIS IN THE MONKEY.

By HERBERT D. TAYLOR, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, October 30, 1918.)

When poliomyelitic virus has once been established in the monkey, it becomes more virulent for this animal by repeated passage.[1] Hence the virus used in experimental work usually brings about a severe form of the disease and high death rate. Occasionally an animal survives the stage of complete prostration if carefully attended and recovers with residual contractures. Experimental poliomyelitis in the monkey, then, is comparable with the severer forms in man, and observations on the variation in leucocyte count in the circulating blood would therefore be expected to yield more nearly uniform results than studies on human cases. In the latter there are many factors of difference in reaction and resistance, age and virulence of the virus, which might contribute to variation. Moreover, opportunities for observations during the incubation period in human cases are rare.

It is generally accepted that abnormal white blood counts are constantly found in poliomyelitis, but opinion is divided on the characteristics of this change.

Müller[2] asserts that a distinct leucopenia with a relative lymphocytosis is characteristic and pathognomonic of the febrile stage, while La Fétra[3] had previously described a moderate leucocytosis as being characteristic of the acute stage in human beings.

Gay and Lucas[4] summarize their blood findings as follows: "The acute stage of anterior poliomyelitis, as it occurs in human beings, and as it is produced experimentally in monkeys, is characterized by the occurrence of a distinct leuko-

[1] Flexner, S., Clark, P. F., and Amoss, H. L., *J. Exp. Med.*, 1914, xix, 195.

[2] Müller, E., Die spinale Kinderlähmung, Berlin, 1910.

[3] La Fétra, L. E., *Arch. Pediat.*, 1909, xxvi, 328.

[4] Gay, F. P., and Lucas, W. P., *Arch. Int. Med.*, 1910, vi, 330.

penia. The differential count shows a relative increase in number of eosinophils and lymphocytes."

Peabody, Draper, and Dochez[5] found in human cases a constant and marked leucocytosis. They also found a constant increase in polymorphonuclear cells of 10 to 15 per cent and a diminution of lymphocytes of 15 to 20 per cent.

OBSERVATIONS.

Blood counts were made on six series of monkeys as follows: Series I, 40 normal monkeys for comparison (Table I); Series II, 4 monkeys already prostrate from experimental poliomyelitis (Table II); Series III, 12 monkeys during the incubation period, later developing the disease; Series IV, 5 monkeys which received the virus but did not develop the disease; Series V, 4 monkeys during the period of recovery from the acute stage; Series VI, 5 monkeys which had passed through the attack and recovered with residual paralyses.

In order to reduce the hourly variations in the blood counts the samples were collected at about the same time on the days of observation between 10.30 and 11.30 a.m.

Series I.—121 counts were made on 40 healthy, adult monkeys (*Macacus rhesus*). Averages of all counts are recorded in Table I.

TABLE I.

Average of 121 White and Differential Blood Counts on 40 Normal Monkeys.

White cells per c.mm.	Lymphocytes.		Polymorphonuclear leucocytes.			Large mononuclear and transitional leucocytes.	Total lymphocytes.		Total polymorphonuclear neutrophilic leucocytes per c.mm.
	Small.	Large.	Neutrophilic.	Eosinophilic.	Basophilic.				
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
22,181	47.5	6.2	41.1	4.1	0.4	0.6	53.7	11,815	9,116

Series II.—Seven counts on four monkeys paralyzed as a result of experimental inoculation with the virus of poliomyelitis are recorded in Table II.

[5] Peabody, F. W., Draper, G., and Dochez, A. R., A clinical study of acute poliomyelitis, Monograph of The Rockefeller Institute for Medical Research, No. 4, New York, 1912, 97.

TABLE II.

White and Differential Blood Counts on Monkeys Prostrate after Poliomyelitic Infection.

Monkey No.	Length of time after inoculation.	Day of prostration.	White cells per c.mm.	Lymphocytes.		Polymorphonuclear leucocytes.			Large mononuclear and transitional leucocytes.	Total lymphocytes.		Total polymorphonuclear neutrophilic leucocytes per c.mm.
				Small.	Large.	Neutrophilic.	Eosinophilic.	Basophilic.				
	days			*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
1	17	2	8,875	13.7	6.3	74.7	2.0	0.0	3.3	20.0	1,775	6,630
2	37	*	9,150	20.0	3.3	76.0	0.3	0.0	0.3	23.3	2,132	6,954
	38	1	8,275	24.3	2.7	70.0	3.0	0.0	0.0	27.0	2,234	5,793
	41	4	4,900	21.6	3.3	73.7	0.7	0.0	0.7	25.0	1,225	3,611
	47	10	4,675	28.3	18.7	53.0	0.0	0.0	0.0	47.0	2,197	2,478
3	10	2	17,550	15.7	1.7	82.7	0.0	0.0	0.0	17.4	3,054	14,514
4	10	3	21,600	14.0	5.7	80.3	0.0	0.0	0.0	19.7	4,255	17,345

* Almost prostrate; both legs flaccid; left arm and back weak.

Monkey 1 of this series received the virus by the nasal route. An intraspinal injection of 2 cc. of normal horse serum had been given on the day preceding the application of the virus to the nasal mucous membrane. After 10 days the monkey became excitable, after 11 days ataxic, and on the 15th day it became prostrate. The blood count was made on the 17th day after infection, which was the 8th day of the disease and the 2nd day after the monkey had become completely prostrated. Death occurred on the 21st day, and autopsy showed well defined lesions of poliomyelitis.

Monkey 2 was inoculated intracerebrally with an incubated mixture of 0.2 cc. of a Berkefeld filtrate of an active glycerolated virus and 2 cc. of human immune serum. The incubation period was unusually long, *viz.* 30 days; the course of the disease was also unusually slow, since the animal did not become prostrate until the 38th day after injection. When the first count was made on the 37th day after injection both legs were flaccid, and the left arm was weak. On the following day, when the animal became prostrate, another count was made. Other counts were made on the 41st and 47th days after injection, which were the 11th and 17th days of the disease. The animal succumbed on the 49th day, and microscopic lesions of poliomyelitis were found in the brain and cord.

Monkeys 3 and 4 were inoculated intracerebrally with an incubated mixture of 0.2 cc. of a Berkefeld filtrate of a 5 per cent suspension of an active glycerolated virus and 2 cc. of streptococcus immune rabbit serum 10 days before the blood

counts were made. Monkey 3 had been prostrate 2 days and Monkey 4 3 days at this time. Both animals were etherized on the 14th day, and autopsy showed characteristic lesions of poliomyelitis.

Series III.—Blood counts were made on twelve monkeys before inoculation with active virus, during the incubation period, and during the acute stage of poliomyelitis. Observations were made at intervals until the animals were killed by etherization or died of respiratory paralysis. All injections of the virus were intracerebral except in Monkeys 8 and 9, which received the virus by the nasal route on the day following an intraspinal injection of 2 cc. of normal horse serum. Nos. 5, 6, and 7 were from the Philippines, monkeys apparently closely similar to *Macacus cynomolgus*. The remainder were *Macacus rhesus*. The results are recorded in Table III. The variations in the circulating lymphocytes in Monkeys 5, 6, 7, and 8 are graphically represented in Text-fig. 1, *a*, *b*, *c*, and *d*, those of Monkeys 9 to 16 in Text-figs. 2 to 4.

Series IV.—Counts on five monkeys (Nos. 17 to 21), which received active virus by several routes but which did not develop the disease, are tabulated in Table IV. Fresh virus was given to Monkey 17 by mouth, to Monkey 18 by means of a nasal plug, to Monkey 19 by intrasciatic injection, and to Monkeys 20 and 21 by intracerebral inoculation.

Series V.—A summary of counts at intervals on monkeys during the acute stage and when partial or complete recovery had taken place is given in Table V. The fluctuations of the circulating lymphocytes are graphically represented in Text-figs. 5 and 6.

Monkey 22 of this series was injected intracerebrally with cultivated virus, first generation,[6] 4 days before the first blood count was made. At this time convulsions and ataxia were noted. On the 7th day after inoculation, when the third white blood count was made, the animal was partially paralyzed, but never became prostrate. 15 days after symptoms were first noted, at the time of the last blood count, the animal had almost completely recovered.

Monkey 23 was exposed to x-rays during the interval between the first and second blood counts, and the blood exhibited the characteristic lymphocytic drop.[7] Seven doses of unfiltered x-rays of 6 Holzknecht units each were given over

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[6] Smillie, W. G., *J. Exp. Med.*, 1918, xxvii, 319.

[7] Taylor, H. D., Witherbee, W. D., and Murphy, Jas. B., *J. Exp. Med.*, 1919, xxix, 53.

TABLE III.

Blood Counts on Monkeys during the Incubation Period after Injections of Poliomyelitic Virus.

Monkey No.	Day of observation.	Length of time after inoculation.	Day of symptoms.	Total lymphocytes.		Total polymorphonuclear neutrophilic leucocytes.	
		days		*per cent*		*per cent*	
5*	1			24.0	6,780	74.7	21,103
	3	2		33.4	6,430	66.0	12,705
	4	3		47.7	7,966	51.7	8,643
	6	5	1	12.7	2,937	86.7	20,049
	7	6	2	44.7	9,834	54.3	11,946
	8	7	3	38.0	6,983	61.0	11,204
	9	8	4	20.7	5,988	79.3	22,938
	11	10	6	15.3	4,777	85.3	26,635
	14	13	9	12.3	3,795	85.3	26,465
6*	1			22.0	6,067	77.3	21,223
	3	2		27.0	3,692	72.3	9,887
	4	3		11.7	2,533	88.3	19,117
7*	1	3		34.7	10,315	61.3	18,221
	2	4		41.0	4,797	47.7	5,581
	4	6	1	10.0	1,948	90.0	17,528
8†	1			49.7	8,225	47.3	7,828
	2			51.0	6,464	45.3	5,742
	5			38.0	6,441	58.3	9,882
	9	4		60.7	8,270	38.3	5,218
	11	6		15.7	4,887	84.3	26,238
	13	8	1	42.3	7,254	56.0	9,604
	14	9	2	30.0	5,670	67.3	12,720
9†	1			47.7	9,922	49.7	10,347
	12	2	11	32.0	5,040	66.0	9,735
	13	3	12	38.4	5,126	60.7	13,230
	14	4	13	27.4	4,226	72.0	11,106
	15	5	14	40.7	4,701	58.0	6,699
	16	6	15	30.0	3,833	68.7	8,776
	18	8	17	30.0	4,583	70.0	10,693
	19	9	18	16.0	2,524	83.7	13,204

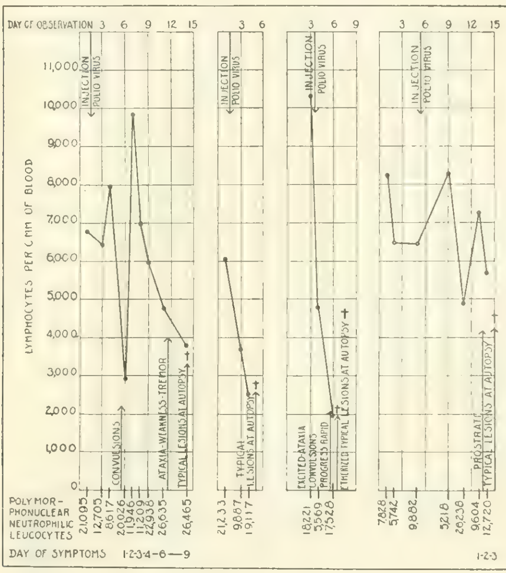
* Philippine monkey.

† Nasal route of infection. Previous intraspinal injection of 2 cc. of normal horse serum.

TABLE III—*Concluded.*

Monkey No.	Day of observation.	Length of time after inoculation.	Day of symptoms.	Total lymphocytes.		Total polymorphonuclear neutrophilic leucocytes.	
		days		*per cent*		*per cent*	
10	1	4		32.0	10,696	54.7	18,214
	3	6		51.5	15,939	47.1	14,578
	5	8	1	36.7	4,936	62.0	8,339
	6	9	2	23.0	3,835	73.7	12,290
	7	10	3	32.0	4,304	64.7	8,702
	8	11	4	29.0	3,567	68.7	8,450
	9	12	5	62.0	6,417	37.3	3,861
	10	13	6	33.7	4,457	66.3	8,768
11	1	2		46.7	12,117	49.3	12,793
	2	3		47.7	9,051	46.7	8,861
	5	6		44.0	8,217	53.7	10,029
	6	7	1	13.0	3,452	86.7	23,019
12	1			57.7	15,348	34.0	9,044
	3	2		60.0	10,740	37.0	6,623
	4	3		78.0	11,661	19.0	2,841
	5	4		69.3	19,477	28.3	3,870
	7	6		64.4	13,347	35.3	7,316
	8	7	1	19.7	6,161	79.7	24,926
13	1			37.3	12,476	62.3	20,839
	3	2		48.7	10,373	49.3	10,501
	6	5		70.7	9,297	28.7	3,744
	7	6		29.7	14,323	69.3	23,420
	8	7		36.7	5,633	63.0	9,671
	9	8	1	18.7	3,675	81.0	15,917
14				38.7	9,298	57.7	13,862
	8	7		22.4	3,489	76.7	11,946
	11	10	2	10.0	2,373	89.3	21,186
	12	11	3	20.0	2,970	80.0	11,880
	13	12	4	14.3	2,431	82.3	13,991
15	1	3		58.7	12,151	40.3	8,342
	4	6		46.7	17,046	51.7	18,871
	7	9	2	24.7	3,557	72.3	10,411
	8	10	3	17.7	3,615	79.3	16,197
16	1	4		45.0	8,179	53.3	9,687
	5	8	1	22.0	2,794	76.7	9,741
	6	9	2	14.7	2,132	83.7	12,137
	7	10	3	13.0	1,940	86.3	12,945
	8	11	4	11.0	2,540	87.0	20,097

a period of 6 days, the dorsal and ventral surfaces of the body being alternately exposed. Each dose was governed by the following factors: spark-gap 3 inches, milliamperes 10, distance from the target of the Coolidge tube to the skin 12 inches, and time of exposure 4 minutes. After these treatments the animal was inoculated intracerebrally with active virus and became completely prostrate



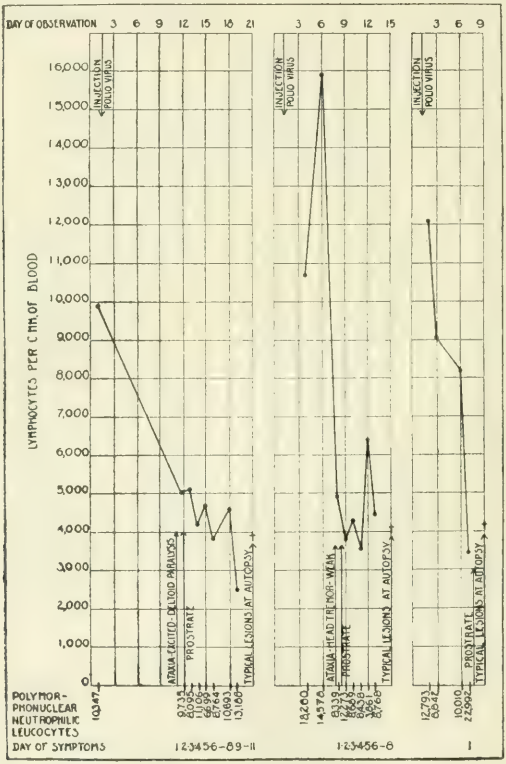
a b c d

TEXT-FIG. 1, *a*, *b*, *c*, and *d*. Lymphocyte curve of Monkeys 5, 6, 7, and 8 (Table III).

8 days later. The animal recovered with marked residual paralysis of both legs. Counts were made on the 59th day of observation, 45 days after prostration, and on the 76th day of observation.

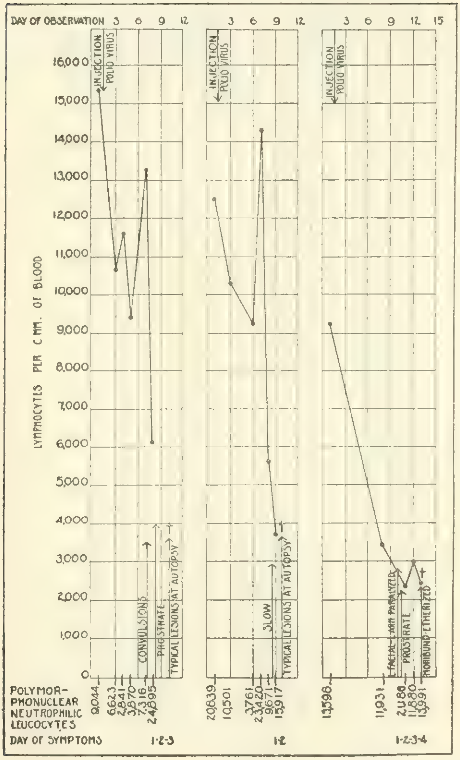
Counts were made on Monkey 24 before injection and 5 days afterward. The animal was prostrate on the 12th day. Counts were made on the 16th, 28th, and 172nd days.

The first count was made on Monkey 25, 16 days after prostration, and the subsequent lymphocytic curve was followed for 22 days, during recovery with residual paralysis.

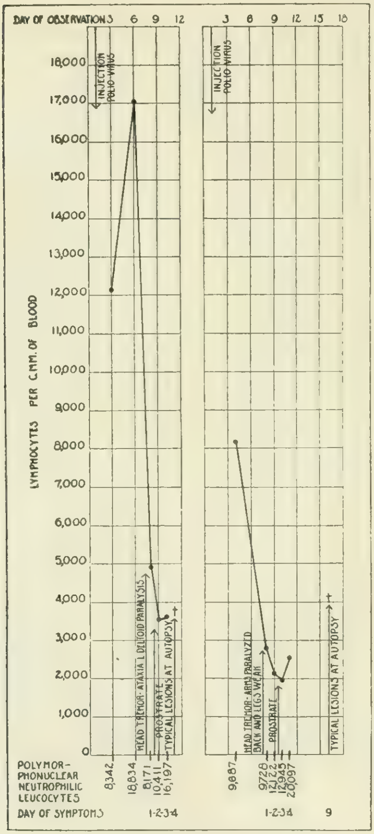


a b c

TEXT-FIG. 2, *a*, *b*, and *c*. (*a*) Lymphocyte curve of Monkey 9 (Table III). (*b*) Lymphocyte curve of Monkey 10 (Table III). (*c*) Lymphocyte curve of Monkey 11 (Table III).



TEXT-FIG. 3, *a*, *b*, and *c*. (*a*) Lymphocyte curve of Monkey 12 (Table III). (*b*) Lymphocyte curve of Monkey 13 (Table III). (*c*) Lymphocyte curve of Monkey 14 (Table III).



a b

TEXT-FIG. 4, *a* and *b*. (*a*) Lymphocyte curve of Monkey 15 (Table III). (*b*) Lymphocyte curve of Monkey 16 (Table III).

TABLE IV.

Blood Counts on Monkeys Which Received Virus but Did Not Develop Symptoms.

Monkey No.	Day of observation.	Length of time after inoculation.	Day of symptoms.	Total lymphocytes.		Total polymorphonuclear neutrophilic leucocytes.	
		days		*per cent*		*per cent*	
17	1			41.0	9,615	57.0	12,367
	6	5		51.0	8,096	46.3	7,360
	13	12		31.4	4,561	64.7	9,398
	23	22		54.4	8,772	35.0	5,644
	37	36		46.7	8,556	45.0	8,556
18	1			50.7	11,205	40.3	8,906
	3	1		56.4	9,292	39.7	6,541
	6	4		49.3	8,849	47.0	8,437
	8	6		55.0	16,871	42.3	12,976
	11	9		68.3	16,871	28.3	6,055
	18	16		56.7	17,393	39.7	12,178
	27	25		55.4	11,426	43.0	8,869
	32	30		59.4	15,622	38.7	10,178
	35	33		47.7	9,922	49.7	10,347
19	1			43.3	9,959	51.7	11,891
	6	4		54.0	10,139	42.0	7,886
	8	6		42.7	7,163	54.7	9,178
	11	9		56.7	9,242	39.0	6,357
	13	11		50.0	7,188	43.3	6,224
	21	19		54.7	6,687	43.0	5,257
	31	29		43.0	7,740	56.3	10,134
	39	37		54.7	8,971	43.0	7,052
20	1			66.0	16,320	23.7	5,824
	3	2		71.3	11,889	26.3	4,386
	4	3		72.0	13,392	21.7	4,036
	5	4		66.0	9,719	27.7	4,079
	7	6		64.0	14,848	30.3	7,030
	8	7		62.0	10,804	31.7	5,524
	9	8		56.7	9,667	34.7	5,916
	11	10		58.0	8,715	36.7	5,514
	15	14		74.3	16,718	20.3	4,568
	18	17		59.3	10,674	36.0	6,480
	22	21		66.7	14,724	25.0	5,519
	26	25		74.3	13,764	18.0	3,334
	33	32		79.7	15,362	16.3	3,142
	36	35		72.3	16,792	23.3	5,178

TABLE IV—*Concluded.*

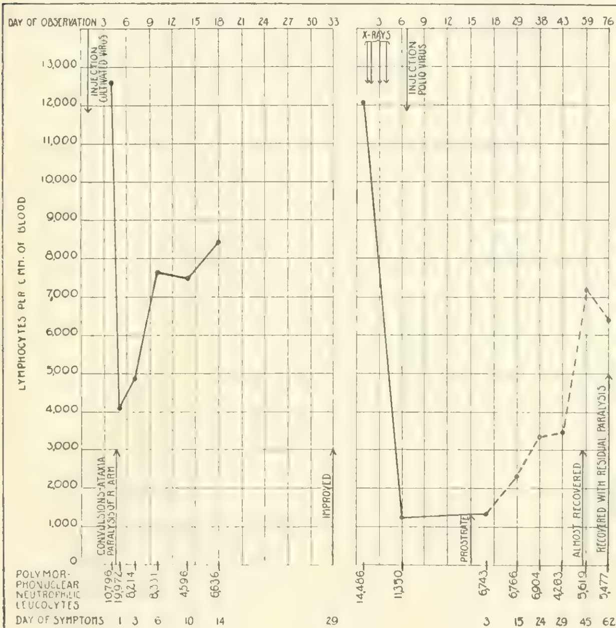
Monkey No.	Day of observation.	Length of time after inoculation.	Day of symptoms.	Total lymphocytes.		Total polymorphonuclear neutrophilic leucocytes.	
		days		*per cent*		*per cent*	
21	1			61.0	11,087	33.0	5,998
	2	1		30.7	4,313	66.3	9,315
	7	6		45.3	6,670	47.7	6,024
	8	7		62.3	7,507	35.0	4,218
	11	10		60.3	9,105	36.7	5,542
	14	13		67.3	11,592	30.7	5,288
	17	16		76.7	10,086	22.7	2,985
	21	20		64.0	10,304	34.0	5,474

TABLE V.

Blood Counts on Monkeys Observed during the Process of Recovery from Poliomyelitis.

Monkey No.	Day of observation.	Length of time after inoculation.	Day of symptoms.	Total lymphocytes.		Total polymorphonuclear neutrophilic leucocytes.		Remarks.
		days		*per cent*		*per cent*		
22	1	4	1	55.3	12,619	45.7	10,820	Convulsions; ataxia.
	2	5	2	16.7	4,087	81.7	11,996	
	4	7	4	36.3	4,864	61.3	8,214	Paralyzed but not prostrate.
	7	10	7	29.3	7,640	70.3	18,331	
	11	14	11	62.3	7,476	38.3	4,596	
	15	18	15	55.0	8,429	43.3	6,636	Almost recovered.
23	1			45.0	12,071	54.0	14,486	X-rayed after count.
	6			9.0	1,231	83.0	11,350	
	17	11	3	16.0	1,332	81.0	6,743	Prostrate 3 days.
	29	23	15	23.3	2,318	68.0	6,766	Partly recovered.
	38	32	24	29.3	3,355	60.3	6,904	
	43	37	29	42.3	3,485	50.3	4,263	
	59	53	45	53.7	7,236	41.7	5,619	Almost recovered.
	76	70	62	50.3	6,363	43.3	5,477	Recovered with residual paralysis.
24	1			65.0	19,565	30.3	10,033	
	6	5		41.7	17,889	56.7	24,324	
	17	16	4	20.0	6,625	79.7	26,401	Prostrate 3 days.
	29	28	16	20.0	7,340	76.3	29,528	
	173	172	160	57.7	15,593	34.7	9,378	Recovered with residual paralysis.
25	1	30	16	29.3	3,289	69.3	7,779	Prostrate.
	2	31	17	53.7	7,357	44.3	6,069	
	10	39	25	56.7	8,661	39.0	5,957	
	15	44	30	67.0	12,462	30.3	5,636	
	22	51	37	41.7	9,570	54.0	12,393	Partial recovery with residual paralysis.

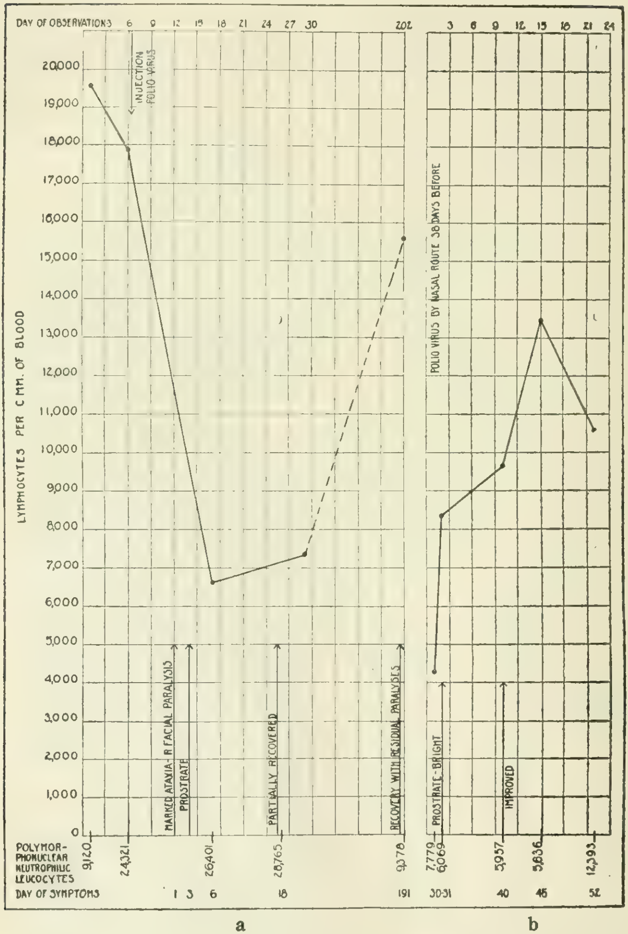
Series VI.—In Table VI are given blood counts made on monkeys which had completely recovered from acute poliomyelitis and proved immune to subsequent inoculations of active virus. All the animals



a b

TEXT-FIG. 5, *a* and *b*. (*a*) Lymphocyte curve of Monkey 22 (Table V). (*b*) Lymphocyte curve of Monkey 23 (Table V).

had some residual contractures. Recovery, in the animals of this group, was of long duration, varying from 1 to 3 months at the time of the first blood count.



TEXT-FIG. 6, *a* and *b*. (*a*) Lymphocyte curve of Monkey 24 (Table V). (*b*) Lymphocyte curve of Monkey 25 (Table V).

TABLE VI.

Blood Counts on Monkeys Which Had Recovered from Acute Symptoms before the First Count.

Monkey No.	Day of observation.	Length of time since recovery.	Total lymphocytes.		Total polymorphonuclear neutrophilic leucocytes.	
		days	*per cent*		*per cent*	
26	1	72	52.7	8,550	43.7	7,090
	2	73	36.3	6,561	61.3	11,080
	5	76	55.0	6,463	40.3	4,735
27	1	72	57.7	7,746	28.0	3,759
	2	73	66.7	7,270	26.3	2,967
	5	76	72.0	7,200	20.0	2,000
28	1	92	50.7	4,700	45.7	4,277
	2	93	38.0	17,733	59.7	12,149
	5	96	43.3	6,712	54.0	8,370
29	1	84	46.3	7,281	47.3	7,438
	2	85	43.3	5,932	53.7	7,357
	6	89	40.3	8,181	52.3	10,617
	8	91	44.0	8,965	51.3	10,452
	9	92	52.7	7,879	42.7	6,384
	16	99	33.7	7,515	61.3	13,670
	20	103	42.3	8,238	50.3	9,796
	35	118	43.3	8,335	52.3	10.068
30	1	31	73.0	16,608	21 7	4,937
	2	32	67.0	10,318	29.0	4,466
	5	35	71.7	16,168	16.7	3,766
	16	46	39.0	14,182	60.3	20,381
	20	50	79.3	24,524	16.3	5,040

DISCUSSION.

All the blood counts made on monkeys during the course of typical acute experimental poliomyelitis show a variation from the normal. This change is apparent in Table VII,[8] in which the average lymphocytes and polymorphonuclear counts and percentage during the acute stage are compared with normal averages. There are included aver-

[8] Counts on Monkey 6 (Table III) are not included in the computation.

ages at the time of the highest and also of the lowest lymphocyte count. The variations are sufficiently great to warrant definite conclusions.

After injection of active poliomyelitic virus the lymphocytes are diminished but return to their former number and are actually increased between the 4th and 6th days of the incubation period (Table III). The polymorphonuclear count is high at this time. The normal average of lymphocytes of 11,815 (Table I) is increased to an average of 19,696 though the average percentage is slightly lowered. During the first 3 days after onset a marked diminution in the lymphocytes takes place. Thus, instead of an average normal lymphocytic count of 11,815, the number is 3,302, and the average percentage

TABLE VII.

Average Variation in White Cells during Infection with Poliomyelitis.

Status of monkeys at time of counts.	No. of animals studied.	Total No. of counts.	Large and small lymphocytes. Average.	Average total No. of lymphocytes per c.mm.	Average total No. of polymorphonuclear neutrophilic leucocytes.	
			per cent		*per cent*	
Normal	40	121	53.7	11,815	41.1	9,116
Incubation period at time of highest lymphocytic count	12	12	44.0	19,696	54.3	13,383
In acute stage at time of lowest lymphocytic count	12	12	15.6	3,302	83.7	18,231
Prostrate	4	6	21.9	2,758	76.5	10,800
Recovered with residual paralysis	6	22	53.2	9,026	41.3	6,800

is 15.6. At this time the polymorphonuclear neutrophilic leucocytes are materially increased in number (18,231) as compared with an average normal of 9,116, and the percentage is also increased from an average of 41 to 84 per cent of the total white cells. When the monkeys become completely prostrate metabolism is at low ebb, and there occurs a further decrease in the actual number of lymphocytes (Table III) which remains low for long periods (Tables II and V). The total number of polymorphonuclear neutrophilic leucocytes returns to normal, but there remains a relative increase averaging 77 per cent. Finally, during recovery both types of cells return to the average normal count and relation. No stimulation of the lympho-

cytes above normal appears during recovery. The counts made on one animal (Monkey 24, Table V) at 160 days after onset are almost the same as those made before the injection of virus.

In the monkeys receiving virus but not developing symptoms there seemed to be a constant tendency for the lymphocytes to decrease following the administration of the virus (Table IV). This decrease was followed by a gradual return to normal.

Monkey 23 (Table V) was x-rayed before inoculation with the virus of poliomyelitis, and the return of the circulating lymphocytes to their normal level, in this animal, seemed to be considerably delayed. As the x-rays have a depleting effect on the circulating lymphocytes[9] similar to that exhibited by the virus of poliomyelitis, the protracted recovery may, in this instance, depend on a summative action of the two agents.

Monkey 22 (Table V) was also included in the series used by Smillie[6] in attempts to produce poliomyelitis with cultivated virus. Typical pathological lesions were not observed at autopsy; however, the symptoms and the blood curve simulating those observed in known poliomyelitic monkeys are suggestive and offer possible additional proof that the monkey developed mild poliomyelitis after intracerebral inoculation with the fourth generation of a culture of the globoid bodies and recovered before it was etherized for autopsy.

SUMMARY AND CONCLUSIONS.

Blood counts were made on six series of monkeys before and at various intervals after the injection of active poliomyelitic virus. From the data thus obtained the variations in the circulating white cells have been followed in the several stages of the disease (*a*) before injection, (*b*) during the incubation period, (*c*) during the acute stage, including the stage of prostration, and (*d*) during recovery. On account of experimental conditions not all the monkeys were observed during the four periods, so that averages of counts in several monkeys are used for comparison.

Averages of 121 counts on 40 normal monkeys are recorded.

[9] Amoss, H. L., Taylor, H. D., and Witherbee, W. D., *J. Exp. Med.*, 1919, xxix, 115.

Immediately following the injection of the virus the relative and actual numbers of lymphocytes are slightly diminished. In many cases the curve continues sharply downward. In others from the 4th to the 6th day there is an actual increase for a brief period to a point somewhat in excess of the original count. With the onset of symptoms a lymphocytic crisis takes place. The curve then continues slightly downward, while the polymorphonuclear neutrophilic leucocytes are relatively and actually increased at approximately the same time. During the stage of prostration the curve of the polymorphonuclear neutrophilic leucocytes returns to almost normal, while the lymphocytic curve continues slightly downward. With recovery the lymphocytes slowly return to normal after several weeks. There is no evidence of lymphocytic stimulation after recovery.

Eosinophilic, basophilic, large mononuclear, and transitional leucocytes follow the depressions and stimulations exhibited by the neutrophilic cells of the same series.

The results here recorded are consistent with the observations of Peabody, Draper, and Dochez on human cases.

The increase in the total number of circulating lymphocytes after the lymphocytic crisis is coincident with the passing of the acute stage.

Additional evidence is presented to indicate that Smillie produced atypical but definite poliomyelitis in the monkey with cultivated virus.

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EFFECTS OF LARGE DOSES OF X-RAYS ON THE SUSCEPTIBILITY OF THE MONKEY TO EXPERIMENTAL POLIOMYELITIS.

By HAROLD L. AMOSS, M.D., HERBERT D. TAYLOR, M.D., AND WILLIAM D. WITHERBEE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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The ready communicability of poliomyelitis among human beings is generally accepted save by a certain few who disregard the idea of selective susceptibility. It is a matter of common observation that of many persons of the most susceptible age definitely exposed only a small percentage contracts the disease. For example, in the New York City epidemic of 1916 there were approximately 1,200,000 individuals of the more susceptible ages, *i.e.* less than 16 years,[1] exposed, but only 8,750 of this age in a total of 9,023 cases were reported. In the epidemic in Westchester County single cases in families of several children were the rule, although in uncommon instances there were three or four cases in the same family. This contrasts strongly with the experience in measles, a disease of practically no selective action. The reasons for the differential power of poliomyelitis to attack are not apparent; however, three facts have been lately adduced which may have some bearing on the problem. In the first place, it has been shown[2] that the route of infection with the virus by the blood stream in monkeys, which is ordinarily closed, except when massive doses are used, can be traversed by smaller doses after intraspinal injection of substances setting up an aseptic meningitis. Infection by the nasal route is also rendered much easier by similar preparation. Flexner and Amoss[3] suggest that possibly one reason for infection or non-

[1] The epidemic of poliomyelitis (infantile paralysis) in New York City in 1916, Department of Health of the City of New York, Monograph series, No. 16, New York, 1917.

[2] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1914, xx, 249.

[3] Flexner and Amoss, *J. Exp. Med.*, 1917, xxv, 525.

infection in persons exposed may lie in the patency or continuity, as the case may be, of the meningeal-choroidal complex. Secondly, Zingher[4] has shown that whereas only 30 per cent of apparently normal children yield a positive reaction to intradermal injection of diphtheria toxin, about 80 per cent of persons recently recovered from poliomyelitis give a positive reaction. This suggests either a general state of lowered resistance, as indicated by susceptibility to both poliomyelitis and diphtheria, or less probably that infection with the former reduces resistance to the latter. Thirdly, Amoss and Taylor[5] noted the power of nasal washings from certain individuals to neutralize the virus of poliomyelitis and suggest that this action may be the first line of defense against poliomyelitic infection.

No definite data have been presented to indicate variations in resistance to poliomyelitic virus among monkeys, except perhaps the impression that tuberculous animals seem to be more susceptible. Moreover, the conditions of the experimental infection are purposely arranged to reduce the chance of variations in individual resistance. The virus when it has once been adapted to the monkey becomes highly infective and remains so during many animal transfers. Finally, however, after several years passage the virulence diminishes.[6] The original M. A. strain adapted to monkeys by Flexner and Lewis[7] in 1909 now possesses less infective power and lends itself to observations on susceptibility in experimental poliomyelitis.

The observations of Peabody, Draper, and Dochez[8] on human cases and of Taylor[9] in experimental poliomyelitis with regard to the remarkable lymphocytic changes accompanying the infection and the focal infiltrations of these cells as one of the histological characteristics of the disease suggest an intimate relation between the infection and the circulating lymphocytes. It may be possible by bring-

[4] Zingher, A., *Dept. Health, City of New York, Reprint Series, No. 52*, 1917.

[5] Amoss, H. L., and Taylor, E., *J. Exp. Med.*, 1917, xxv, 507.

[6] Flexner, S., Clark, P. F., and Amoss, H. L., *J. Exp. Med.*, 1914, xix, 195.

[7] Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1909, liii, 1639.

[8] Peabody, F. W., Draper, G., and Dochez, A. R., A clinical study of acute poliomyelitis, Monograph of The Rockefeller Institute for Medical Research, No. 4, New York, 1912.

[9] Taylor, H. D., *J. Exp. Med.*, 1919, xxix, 97.

ing about conditions in which the lymphocytes are greatly diminished to render the monkey more susceptible to poliomyelitic virus. The experiments here recorded support this belief.

EXPERIMENTAL.

By exposure to properly controlled doses of x-rays it is possible to diminish the circulating lymphocytes.[10] Accordingly, two normal monkeys were selected for each experiment. One was treated with x-rays[11] daily or every other day until the total number of lymphocytes per c.mm. of blood was between 1,000 and 2,000. A Berkefeld filtrate of a 5 per cent suspension of glycerolated M. A. virus was injected intracerebrally into the x-rayed and the control animal. The dose of virus was previously titrated and known to be subinfective. Should the dose of the virus be too large, both animals are infected, and if the maximum subinfective dose is reduced by more than one-half, the more susceptible x-rayed animal does not respond with an attack of poliomyelitis.

Three series of experiments were carried out according to the plan outlined. The protocols follow.

Series I.—Macacus rhesus A (control). Jan. 17, 1917. Blood count, 7,750 lymphocytes and 3,950 polymorphonuclear neutrophilic leucocytes per c.mm. Injected intracerebrally with 1 cc. of a Berkefeld filtrate of a 5 per cent suspension of glycerolated poliomyelitic monkey cord (M. A. virus). The monkey remained well.

Macacus rhesus B. Jan. 3, 1917. Blood count, 6,930 lymphocytes and 8,465 polymorphonuclear neutrophilic leucocytes per c.mm. Received seven doses of x-rays, 6 Holzknecht units each. Jan. 15. Blood count, 1,257 lymphocytes and 4,300 polymorphonuclear neutrophilic leucocytes per c.mm. (Text-fig. 1, *a*). Jan. 17. Received intracerebrally (at the same time as the control injection) 1 cc. of the Berkefeld filtrate of M. A. virus used in Monkey A. Jan. 23. Excitable; left arm paralyzed; right deltoid weak. Jan. 24. Prostrate. Jan. 27. Moribund; etherized.

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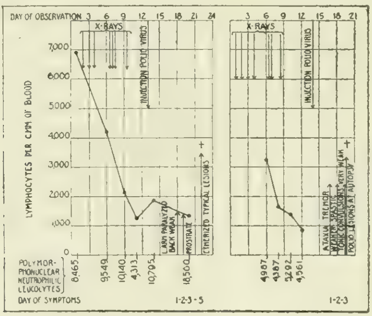
[10] Taylor, H. D., Witherbee, W. D., and Murphy, Jas. B., *J. Exp. Med.*, 1919, xxix, 53.

[11] Unfiltered x-rays of 6 Holzknecht units each were given at each dose alternately on the dorsal and ventral surfaces. Each dose was governed by the following factors: spark-gap 3 inches, milliamperes 10, distance from the target of the Coolidge tube to the skin 12 inches, and time of exposure 4 minutes.

Autopsy.—Severe acute lesions of poliomyelitis, perivascular infiltration, and necrosis of ganglion cells.

Series II.—*Macacus rhesus* C (control). Feb. 5, 1917. Blood count showed 16,630 lymphocytes and 4,050 polymorphonuclear neutrophilic leucocytes per c.mm. Injected intracerebrally with 1 cc. of a Berkefeld filtrate of a 5 per cent suspension of glycerolated poliomyelitic monkey cord (M. A. virus). The monkey remained well. Mar. 5. Began daily treatments with x-rays. After seven doses the lymphocytes were reduced in number to 878 per c.mm., but no symptoms of poliomyelitis developed. 6 months later the animal showed symptoms of tuberculosis and died.

Autopsy.—Marked tuberculosis of lungs and abdominal viscera.



a b

TEXT-FIG. 1, *a* and *b*. (*a*) Lymphocytic curve of Monkey B, Series I. (*b*) Lymphocytic curve of Monkey D, Series II.

Macacus rhesus D. Blood was not counted before treatment with x-rays. On Feb. 5, 1917, seven doses of x-rays had been given, and the blood count was as follows: lymphocytes 885 (15 per cent), polymorphonuclear neutrophilic leucocytes 4,561 (77.3 per cent) (Text-fig. 1, *b*). On this date there was injected intracerebrally 0.75 cc. of the Berkefeld filtrate of M. A. virus given Monkey C (control). Feb. 8. Ataxic; protects right leg; marked head tremor. Feb. 9. Weaker; ataxia increased. Feb. 10, a.m. Back and both legs weak; convulsion lasting 2 minutes. 2 p.m. Found dead.

Autopsy.—All the organs very pale; spleen very small. Brain and cord showed intense congestion of gray matter. Microscopic examination showed focal infiltrations and perivascular infiltration of cells, most of which were polymorphonuclear leucocytes. The lesions in the cord and spinal ganglia were not marked.

Macacus rhesus E. Blood count before exposure to x-rays, 8,785 (43.6 per cent) lymphocytes and 8,180 (40.6 per cent) polymorphonuclear neutrophilic leucocytes. Received eight doses of x-rays. Feb. 5, 1917. The blood count was as follows: 581 (9 per cent) lymphocytes and 4,810 (74.6 per cent) polymorphonuclear leucocytes. Injected intracerebrally with 0.5 cc. of the poliomyelitic virus filtrate. The animal never showed symptoms of poliomyelitis.

In this experiment the control showed no symptoms after receiving 1 cc. of the virus filtrate, while the animal treated with x-rays, receiving only 0.75 cc., succumbed to poliomyelitis. The control animal after 28 days was treated with x-rays, but no symptoms developed, indicating the destruction or removal of the virus in that time.

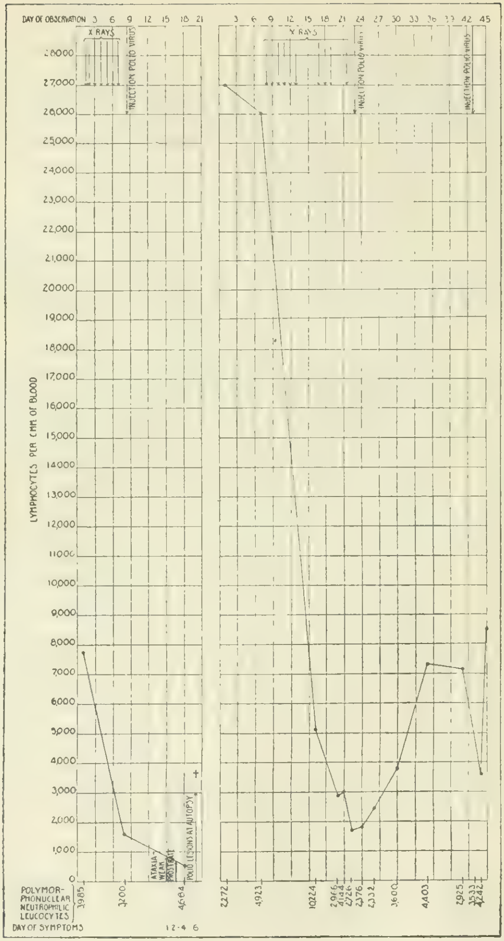
Series III.—Macacus rhesus F (control). Apr. 3, 1918. Blood count, lymphocytes 8,432 (42 per cent) and polymorphonuclear neutrophilic leucocytes 11,162 (55.6 per cent) per c.mm. Injected intracerebrally with 0.5 cc. of a Berkefeld filtrate of a 5 per cent suspension of glycerolated poliomyelitic monkey cord (M. A. virus). The animal showed no symptoms of poliomyelitis, but later died of tuberculosis.

Autopsy.—Almost total involvement of left lung and tuberculosis of abdominal viscera. There was no evidence of poliomyelitic lesion in the brain or cord.

Macacus rhesus G. Mar. 26, 1918. Blood count, 7,751 (59.7 per cent) lymphocytes and 3,985 (30.3 per cent) polymorphonuclear neutrophilic leucocytes per c.mm. Received daily treatments with x-rays, 6 Holzknecht units each, for 7 days. Apr. 3. Blood count, 1,625 (14.7 per cent) lymphocytes and 3,200 (75.3 per cent) polymorphonuclear neutrophilic leucocytes per c.mm. (Text-fig. 2, *a*). Injected intracerebrally with 0.5 cc. of the Berkefeld suspension of the glycerolated poliomyelitic cord (M. A. virus) given Monkey F. Apr. 10. Ataxic; head tremor; partial paralysis of left arm, right deltoid, and back. Apr. 11. Completely prostrate. Apr. 13. Blood count, 520 (10 per cent) lymphocytes and 4,664 (89.7 per cent) polymorphonuclear neutrophilic leucocytes per c.mm. Apr. 15. Found dead.

Autopsy.—Slight perivascular infiltration and nerve cell degeneration in the medulla and slight focal accumulations of cells, most of which were polymorphonuclear neutrophilic leucocytes.

The outcome of this experiment supports the findings in Series I, in which the control animal remained unaffected by a dose of M. A. poliomyelitic virus which caused complete paralysis in a monkey which had been treated with x-rays with a consequent decrease in the circulating lymphocytes.



a b

TEXT-FIG. 2, *a* and *b* (*a*) Lymphocytic curve of Monkey G, Series III. (*b*) Lymphocytic curve of Monkey I.

Survival of a Subinfective Dose of the Virus in Normal Monkey Brain.

The usual incubation period in experimental poliomyelitis is from 7 to 10 days; however, when a weak virus[6] is used, or in neutralization experiments[5] this period may be prolonged to 30 or 40 days. An incubation period longer than 30 days is unusual. It is presumed that the virus lay dormant for the prolonged period or multiplied slowly. Multiple injections of virus weakened by long storage in 50 per cent glycerol[12] apparently lessen rather than increase the resistance of the monkey to subsequent injections, if the interval between injections is about 7 days. Longer periods have not been studied. In monkey poliomyelitic brain removed from the body and placed under anaerobic conditions at 37°C. in tissue ascitic fluid the virus may survive without multiplication for 20, but not for 30 days.[13] The virus does not survive 7 days *in vivo* in rabbit brain[14] or even for 2 days in the rat brain.[15] Is the subinfective dose then disposed of in a monkey of normal resistance within the maximum incubation period? The following experiments have a bearing on this question.

Experiment 1.—Monkey C, used as control in Series II, received 1 cc. of virus filtrate intracerebrally. When 28 days had elapsed the animal was exposed to x-rays until the lymphocytes had decreased to 878 per c.mm. The animal had no symptoms of poliomyelitis.

Experiment 2.—*Macacus rhesus* H. Mar. 23, 1917. Received 1 cc. of filtrate of suspension of virus used in Experiment 1 and at the end of 15 days was exposed to x-rays, which reduced the lymphocytes to 1,125 per c.mm. No symptoms of poliomyelitis developed in the animal.

Apparently in the animals of normal resistance subinfective doses are disposed of in 15 days or are so weakened that reduction in resistance of the animal does not permit of infection. The experiments were not carried further because of lack of monkeys due to war conditions.

[12] Flexner and Amoss, *J. Exp. Med.*, 1917, xxv, 539.

[13] Flexner and Amoss, *J. Exp. Med.*, 1915, xxi, 509.

[14] Amoss, H. L., *J. Exp. Med.*, 1918, xxvii, 443.

[15] Amoss, H. L., and Haselbauer, P., *J. Exp. Med.*, 1918, xxviii, 429.

Is Immunity Once Established Reduced by Exposure to X-Rays?

Flexner and Lewis[16] have shown that monkeys which have passed through an attack of poliomyelitis are immune to subsequent injections of an active virus. Moreover, sera from immune monkeys and from human beings[17] who have had the disease possess definite neutralizing power for the poliomyelitic virus. It is probable that the immunity could not be destroyed by any of the factors accompanying exposure to x-rays. A single experiment was carried out on a monkey paralyzed by previous injections of poliomyelitic virus.

Experiment 3.—Macacus rhesus I (active immune) had received dried poliomyelitic virus on Feb. 10, 1917, showed symptoms on Feb. 21, and had complete paralysis of both legs and deltoid muscles on Feb. 23. The animal gradually improved with residual paralysis of both legs. Apr. 6. Blood count, 26,062 (81 per cent) lymphocytes and 4,920 (15.3 per cent) polymorphonuclear neutrophilic leucocytes per c.mm. X-ray treatments were begun. During the succeeding 14 days ten exposures, of 6 Holzknecht units each, were made. Apr. 24. The blood count was as follows: 1,739 (37 per cent) lymphocytes and 2,726 (58 per cent) polymorphonuclear neutrophilic leucocytes per c.mm. Apr. 25. Injected intracerebrally with 1 cc. of a 5 per cent suspension of fresh poliomyelitic monkey cord. No symptoms developed. May 15. Injected intracerebrally with 2 cc. of 5 per cent suspension of active poliomyelitic monkey cord. The animal remained as before without any increase in paralysis or other symptoms of poliomyelitis. The lymphocytic changes are graphically recorded in Text-fig. 2, *b*.

In this experiment exposure to x-rays sufficient to reduce the circulating lymphocytes to one-fifteenth of their original number failed to destroy immunity in the monkey established by a previous attack of poliomyelitis.

SUMMARY AND CONCLUSIONS.

In two series of the experiments here recorded the monkey which had been repeatedly exposed to x-rays responded with typical acute poliomyelitis to an intracerebral inoculation of poliomyelitic virus fil-

[16] Flexner and Lewis, *J. Am. Med. Assn.*, 1910, lv, 662.

[17] Levaditi and Landsteiner, *Compt. rend. Soc. biol.*, 1910, lxviii, 311. Römer, P. H., and Joseph, K., *Münch. med. Woch.*, 1910, lvii, 568. Flexner and Lewis, *J. Am. Med. Assn.*, 1910, liv, 1780. Anderson, J. F., and Frost, W. H., *J. Am. Med. Assn.*, 1911, lvi, 663.

trate, whereas the normal control receiving the same dose showed no symptoms. In another series the x-rayed animal came down with typical poliomyelitis after inoculation with three-fourths of the dose which was not infective for the control. It has been demonstrated that the x-rays diminished both the number of circulating lymphocytes and the resistance of the animal to the weak poliomyelitic virus. Whether the lowered resistance of the animals as the result of the treatment with x-rays is due to the reduction of circulating lymphocytes in each of the x-rayed monkeys is not determined in these experiments.

However, the great reduction in lymphocytes in human cases[8] and in monkeys during the acute stage of experimental poliomyelitis[9] and the gradual return of the cells to their former numbers during recovery strongly suggest a definite relation between these cells and one factor of resistance in poliomyelitis.

On the other hand, the reduction in resistance by x-rays, while definite, is not sufficiently great to warrant the conclusion that we are dealing with major factors governing infection or non-infection.

Another experiment in this paper deals with the survival of a subinfective dose of the virus in the normal monkey brain. A monkey receiving the subinfective dose of the virus was exposed to x-rays at 28 days, another at 15 days after injection, but neither animal showed symptoms of poliomyelitis. It is concluded that within this period the virus did not remain unchanged in the normal monkey brain.

An attempt to reduce the immunity in a monkey acquired by an attack of experimental poliomyelitis was unsuccessful.

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THE ACTION OF CHLORINATED ANTISEPTICS ON BLOOD CLOT.

By HERBERT D. TAYLOR, M.D., and MARIANNE G. STEBBINS.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Experiments recently reported from this laboratory[1] demonstrated that hypochlorite solutions, in the concentration and alkalinity used clinically, exhibit a solvent action on necrotic tissue. Fiessinger and his coworkers[2] have reported a similar action of these solutions on pus cells. The importance of the latter observation is emphasized by the experiments of Rous and Jones,[3] which demonstrated that intact leucocytes may protect bacteria which they have phagocyted from the action of an antiseptic. These bacteria, when liberated after autolysis of the leucocytes, are viable and may proliferate in an apparently normal manner.

As the hypochlorite solutions are extensively used in the treatment of infected wounds, and as their cleansing properties have been emphasized, it seemed important to determine definitely whether these solutions were effective in dissolving or penetrating blood clot. The possibility that clotted blood in wounds might serve as a protective covering for virulent microorganisms, thereby preventing the bactericidal action of the antiseptic employed, seemed worthy of investigation.

As it is generally recognized, and as early experiments seemed to demonstrate conclusively, that solutions of a degree of concentration and alkalinity compatible with clinical use exhibited little solvent action on blood clot, certain modifications were later introduced in an indeavor to influence the solutions in this direction.

[1] Taylor, H. D., and Austin, J. H., *J. Exp. Med.*, 1918, xxvii, 155. Austin, J. H., and Taylor, H. D., *ibid.*, 627.

[2] Fiessinger, N., Moiroud, P., Guillaumin, C.-O., and Vienne, G., *Ann. méd.*, 1916, iii, 133.

[3] Rous, P., and Jones, F. S., *J. Exp. Med.*, 1916, xxiii, 601.

Method.—Rabbit blood was allowed to clot in test-tubes, and after separation from the serum the remaining cylindrical mass was cut into a number of discs, each 1 cm. in thickness and 1.5 cm. in diameter. Each disc was then placed in 50 cc. of the solution to be tested for varying lengths of time. The appearance and size of the discs were noted before and after exposure to the action of the solution and examined carefully for any evidence of dissolution or penetration by the antiseptics. Careful controls were included in every experiment.

EXPERIMENTAL.

Experiment 1.—The first series of experiments was of a morphological nature, and an effort was made to determine by the appearance of the blood clot discs whether or not any solvent action was demonstrable. Dakin's hypochlorite (0.5 per cent sodium hypochlorite), chloramine-T (2 per cent), and dichloramine-T (5 per cent in chlorcosane) solutions apparently had no solvent, disintegrative, or penetrative action on the discs when allowed to act for periods of time varying from 15 minutes to 12 hours.

Experiment 2.—In the hope of varying the permeability of the blood clots, they were first treated with $\frac{M}{8}$ calcium chloride and $\frac{M}{8}$ sodium chloride. Loeb[4] has already shown that calcium chloride increases and sodium chloride decreases the permeability of masses of finely powdered gelatin. After varying lengths of time in the salt solutions (from 15 minutes to 2 hours), the clots were transferred to the antiseptic solutions, hypochlorite 0.5 per cent, chloramine-T 2 per cent, dichloramine-T 5 per cent in chlorcosane, and in distilled water, and allowed to remain in contact with these solutions for from 15 minutes to 12 hours. No appreciable action was noticeable except all absence of hardening of the surface layer of the clot first treated with calcium chloride and later transferred to Dakin's hypochlorite solution. It was then decided to test the penetration of the solutions in a manner allowing objective analysis, and the following experiments were performed in the hope of getting accurate and comparable figures with regard to the relative solvent powers of certain antiseptics.

Experiment 3.—10 cc. of sterile rabbit blood were placed in a sterile test-tube containing 0.5 cc. of a 24 hour bouillon culture of *Staphylococcus aureus* and shaken thoroughly against a sterile rubber stopper to insure even distribution of the bacteria through the resulting clot. After clotting and separation of the serum the cylindrical mass was cut, with precautions against further contamination, into equal sized discs, 1 cm. in thickness and 1.5 cm. in diameter. The discs were then placed in bottles containing 50 cc. of the following solutions: (1) Dakin's hypochlorite solution 0.5 per cent; (2) chloramine-T solution 2 per cent; and (3)

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[4] Loeb, J., *J. Biol. Chem.*, 1917, xxxi, 343.

sterile salt solution, as control. A disc was removed from each bottle after a half hour and a second after a 1 hour interval. The discs were then washed in two changes of sterile saline solution to remove all traces of the test solution; the second saline wash contained a few drops of a $\frac{N}{10}$ sodium thiosulfate solution to neutralize any chlorine remaining. Each disc was thoroughly ground in a sterile mortar with 5 cc. of saline solution, agar tubes were inoculated with two loopfuls of the resulting emulsion, and plates poured. After 24 hours incubation at 37°C. the colonies developing in the plates were counted, and the results are summarized in Table I.

TABLE I.

Time of contact.	Dakin's solution.	Chloramine-T solution.	Salt solution control.
hrs.			
½	60	100	Confluent.
1	60	100	"

Experiments 4 and 5.—These experiments were similar to Experiment 3, and the results are recorded in Tables II and III.

TABLE II.

Time of contract.	Dakin's solution.	Chloramine-T solution.	Salt solution control.
hrs.			
½	300	312	Confluent.
1	280	Confluent.	"
2	Confluent.	300	"

TABLE III.

Time of contact.	Dakin's solution.	Chloramine-T solution.	Salt solution control.
hrs.			
1	Confluent.	Confluent.	Confluent.
2	"	"	"

Experiment 6.—Discs prepared as in Experiments 3 to 5 were placed in $\frac{M}{8}$ calcium chloride and $\frac{M}{8}$ sodium chloride for from 1 to 2 hours and later transferred to Dakin's solution to test the effect of the saline solution on the permeability of the clot. The results are given in Table IV.

TABLE IV.

Solution 1.	Time of contact.	Solution 2.	Time of contact.	No. of colonies.
	hrs.		*hrs.*	
M/8 calcium chloride.	1	Dakin's hypochlorite.	2	222
M/8 " "	1		0	Confluent.
Dakin's hypochlorite.	2		0	800
M/8 sodium chloride.	1	Dakin's hypochlorite.	2	545
M/8 " "	1		0	Confluent.

Experiment 7.—The same experiment was repeated, but in one series (A) the clot discs nearest the top of the clotted blood were used and in the other (B) the clot discs nearest the bottom were used. This was done in order to rule out, as far as possible, inaccuracies due to the uneven distribution of the bacteria in the cylindrical clot. The results of both series are given in Table V.

TABLE V.

Solution 1.	Time of contact.	Solution 2.	Time of contact.	No. of colonies.	
				A	B
	hrs.		*hrs.*		
Dakin's hypochlorite.	2			15	20
M/8 calcium chloride.	1	Dakin's hypochlorite.	2	20	40
M/8 sodium "	1	" "	2	10	40
9 per cent sodium chloride (normal).	3			10	

Experiment 8.—To insure still further even distribution of the bacteria through the blood clot the following variation was instituted. A rabbit was injected in-

TABLE VI.

Solution 1.	Time of contact.	Solution 2.	Time of contact.	No. of colonies.
	hrs.		*hrs.*	
M/8 sodium chloride.	2			Confluent.
M/8 calcium "	2			"
Dakin's 0.5 per cent hypochlorite.	2			"
M/8 sodium chloride.	1	Dakin's 0.5 per cent hypochlorite.	2	"
M/8 calcium "	1	Dakin's 0.5 per cent hypochlorite.	2	"

travenously with 15 cc. of a 24 hour bouillon culture of *Staphylococcus aureus* and 1 minute later bled from the heart. The blood was allowed to clot in the usual manner and discs were prepared as described above. The discs were placed in solutions, taken out, ground as described above, inoculated into agar tubes, and later poured in plates, as in previous experiments. The results are shown in Table VI.

Experiments 9 and 10.—The same experiment was repeated on two later occasions with the results shown in Tables VII and VIII.

TABLE VII.

Solution 1.	Time of contact.	Solution 2.	Time of contact.	No. of colonies.
	hrs.		*hrs.*	
M/8 sodium chloride.	2			50
M/8 calcium "	2			200
Dakin's 0.5 per cent hypochlorite.	2			50
M/8 sodium chloride.	1	Dakin's 0.5 per cent hypochlorite.	2	100
M/8 calcium "	1	Dakin's 0.5 per cent hypochlorite.	2	50

TABLE VIII.

Solution 1.	Time of contact.	Solution 2.	Time of contact.	No. of colonies.
	hrs.		*hrs.*	
M/8 sodium chloride.	2			Confluent.
Chloramine-T 2 per cent.	2			"
Dakin's 0.5 per cent hypochlorite.	2			"
Eusol (0.5 per cent hypochlorite).	2			"
M/8 sodium chloride.	1	Chloramine-T 2 per cent.	2	"
M/8 " "	1	Dakin's 0.5 per cent hypochlorite.	2	"
M/8 " "	1	Eusol (0.5 per cent hypochlorite).	2	"

Experiment 11.—Equal sized discs of blood clot, through which *Staphylococcus aureus* had been equally distributed by the *intra vitam*, intravenous injection of the organisms in the usual manner, were placed first in a trypsin solution and later in Dakin's solution to determine whether the trypsin, by partial disruption of the

clot, rendered it more permeable to the antiseptic solution. A 1 per cent solution of commercial trypsin and Dakin's solution with a hypochlorite concentration of 0.5 per cent were used. The results of this experiment are given in Table IX.

TABLE IX.

Solution 1.	Time of contact.	Solution 2.	Time of contact.	No. of colonies.
	hrs.		*hrs.*	
Trypsin 1 per cent.	2	Dakin's solution.	2	Confluent.
" 1 " "	24	" "	2	"
Saline solution.	2			"

Inasmuch as blood clot had been found so resistant to hypochlorite solutions which readily dissolve necrotic tissue, pus cells, and other organic matter, a series of experiments was performed to determine, if possible, the resistant constituent of the clot. The results are summarized in the description of Experiment 12.

Experiment 12.—(*a*) Plasma clot was prepared by centrifuging at high speed freshly drawn rabbit blood. The resulting supernatant, clear fluid quickly coagulated. This clot, without corpuscles, is readily dissolved by Dakin's hypochlorite solution having a sodium hypochlorite concentration of 0.5 per cent. 2 per cent chloramine-T solution is without such action.

(*b*) Red blood corpuscles, prepared by defibrinating freshly drawn rabbit blood and adding sufficient saline solution to make a 5 per cent suspension of cells, are readily dissolved by the hypochlorite solution. The chloramine-T solution merely laked the corpuscles without exhibiting solvent action.

(*c*) Pus cells, prepared from human exudate and from dogs in response to the irritant action of aleuronat, are dissolved by the hypochlorite but not by the chloramine-T solution.

(*d*) It seemed probable that the fibrin of blood clot was more resistant than the fibrin of the plasma clot because the fibrin is held together in closer mechanical mass by the blood cells than in the more loosely formed plasma.[5]

[5] Experiments were undertaken but proved inconclusive and were interrupted by Dr. Taylor's death.

SUMMARY.

This work demonstrates that the chlorinated antiseptics have no power to penetrate blood clots and destroy bacteria therein contained. Correspondingly, blood clots may protect virulent bacteria for a long period of time and the organisms properly planted will be able to proliferate in a normal manner.[6,7]

[6] It seems probable that the fibrin of the blood clot is the resistant substance as plasma and red and white cells are easily dissolved by these antiseptics.

[7] The work on this paper up to this point had been written up by Dr. Taylor before his death. No attempt has been made to discuss his clear-cut and conclusive experiments. The summary given here is taken from the quarterly report rendered to the Director of The Rockefeller Institute.

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THE TROPISTIC ACTION OF BLOOD VESSELS ON THE MIGRATION OF CHROMATOPHORES.

By HERBERT D. TAYLOR, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 29, 1918.)

Loeb[1] has shown that the chromatophores appearing in the yolk sac early in the development of ***Fundulus heteroclitus*** are attracted to the blood vessels when there is an active circulation. Thus the little red and black cells, each with many ramifying processes, begin to migrate from their scattered positions in the yolk sac to the blood vessels of this organ only after the active heart is propelling the blood through the vessels. If potassium chloride, in quantities sufficient to stop the heart beat and circulation, is added to the water containing the developing embryos, the chromatophores develop normally, but until the 4th day, at least, there is no migration comparable with that observed in controls where by this time the pigment cells are definitely approaching the vessels. Loeb suggests that there is probably something present in the circulating blood which is responsible for this tropistic phenomenon.

The experiments recorded below were devised for the purpose of arriving at a satisfactory explanation of the phenomenon, if possible.[2]

Experiment 1.—60 eggs of *Fundulus heteroclitus*, artificially fertilized, were kept in sea water. After 4 days incubation black and red chromatophores, with widely spread out processes were scattered over the yolk sac. By this time the hearts had begun to beat and the blood was circulating through the thin walled blood vessels which formed a network over the surface of the yolk sac. None of the chromatophores showed any relation to the vessels at this time. On the 6th day of incubation, however, the chromatophores had approached and embraced the blood vessels of the yolk sac so that the location of the vessels could be accurately determined by the coloring of their periphery caused by the closely

[1] Loeb, J., *J. Morphol.*, 1893, viii, 161; On the heredity of the marking in fish embryos, Biological lectures, Boston, 1899, 227.

[2] These experiments were suggested by Dr. Jacques Loeb.

approximated black and not quite so closely approximated red chromatophores. Twenty of the eggs were allowed to remain in the sea water, twenty transferred to $\frac{M}{8}$ potassium chloride in sea water, and twenty to $\frac{M}{16}$ potassium chloride in sea water. The results are summarized in Table I.

TABLE I.

Length of time after fertilization.	Sea water.		M/16 KCl		Transferred from M/16 KCl to sea water.*		Transferred from M/8 KCl to sea water.*	
	No. with active circulation. (Chromatophores on vessels.)	No. with inactive circulation. (Chromatophores spread.)	No. with active circulation. (Chromatophores on vessels.)	No. with inactive circulation. (Chromatophores spread.)	No. with active circulation. (Chromatophores on vessels.)	No. with inactive circulation. (Chromatophores spread.)	No. with active circulation. (Chromatophores on vessels.)	No with inactive circulation. (Chromatophores spread.)
days	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
6	100	0	100	0				
8	100	0	100	0				
9	100	0	45	55				
10	100	0	50	50	60	40	0	100
11	100	0	10	90	40	60	0	100
16	All hatched swimming.		0	100	55	45	10	90
17			0	100	30	70	10	90
18			0	100	45	55	10	90
19			0	100	33	67	20	80
20			0	100	33	67	10	90
25			All dead.		55	45	0	100
26					45	55	0	100
27					70	30	0	100
41					100	0	All dead.	
46					0	100		

* Left in $\frac{M}{8}$ potassium chloride and $\frac{M}{16}$ potassium chloride for 4 days (from 6th to 10th day of incubation period) and then placed in sea water.

The chromatophores of all the embryos developing in sea water alone remained in close contact with the blood vessels until the time of hatching (16th day after fertilization). All hatched, and the fish were apparently normal.

In those left in $\frac{M}{16}$ potassium chloride the hearts and circulations gradually became inactive and as this occurred the chromatophores again became spread out on the surface of the yolk sac. None of these embryos ever hatched. Ten of the latter after 4 days in the $\frac{M}{16}$ potassium chloride (from the 6th to the 10th day of incubation)

were again returned to sea water without excess potassium. At that time 60 per cent still had active hearts and circulations, and the chromatophores were closely embracing the vessels. In 40 per cent the hearts had stopped, the blood was not circulating, and the chromatophores were spread out over the surface of the yolk sac. At the observations made after this time there were at various times different percentages of embryos with active hearts and circulations but always those in which the blood was flowing through the vessels also had the chromatophores in close contact with the blood vessels. Contrasting with this the chromatophores were always spread out over the surface of the yolk sac when the circulation was inactive. On the 41st day of incubation all had active circulation and chromatophores closely approximating the blood vessels. Thus on the 46th day of incubation, which was as far as the observations were continued, 100 per cent showed spread out chromatophores, and in none of these was there an active circulation.

In those left in $\frac{M}{8}$ potassium chloride and later transferred to sea water without excess potassium chloride a few again developed active circulations and in these the chromatophores again approximated and embraced the vessels in the characteristic manner. In those with inactive circulation, however, the chromatophores remained scattered and showed no relation to the blood vessels. When the circulation again stopped, in those that had begun a second time, the chromatophores again became spread out in the same manner.

A few confirmatory observations of interest may be briefly considered here.

1. When the circulation had only been inactive for 24 hours or less as in the column headed "Transferred from $\frac{M}{16}$ KCl to sea water," on the 11th day of incubation, it could be seen that the embryos in which the circulation had last stopped showed a much less pronounced wandering and spreading over of the chromatophores than those in which it had not started again.

2. The result was similar when the circulation had started but 24 hours or less before observation, in the column headed "Transferred from $\frac{M}{16}$ KCl to sea water," on the 18th day of incubation. Thus two of the three embryos in this category showed only partial approximation of the chromatophores to the vessels and not the complete stage noted in the remainder.

3. Sometimes there was an embryo present whose heart gave an occasional spasmodic beat, in the column headed "$\frac{M}{16}$ KCl," on the 11th day of incubation, and in these the chromatophores had not migrated far from the blood vessels and became now partially spread out. This is what would be expected if, as seems probable, this was the last in which the circulation had stopped.

4. In the embryos with beating heart but inactive circulation, in the column headed "Transferred from $\frac{M}{8}$ KCl to sea water," on the 16th day of incubation, there is usually a clump of chromatophores closely approximating the base of the beating heart where it is fixed to the surface of the yolk sac. It seems suggestive that chromatophores collect about the beating heart after they have left the vessels through which the blood has ceased to flow.

5. In other embryos with slow circulation following the removal of the stopped heart from $\frac{M}{8}$ potassium chloride to sea water, we find all stages in the restoration of the circulation and where this is rapid the chromatophores closely approximate the vessels, where slow they form but a loose meshwork about the vessels.

Experiment 2.—This experiment was in every way comparable with Experiment 1.

Experiment 3.—Eggs of the same teleost were allowed to develop normally until the 6th day when they were placed in flasks containing sea water to which had been added a few drops of a dilute solution of sodium cyanide (0.1 per cent). Eggs placed in 50 cc. of sea water to which two drops of the sodium cyanide solution had been added, when removed, at the end of 9 days, still had active circulations. The chromatophores were in about the same state as at the time when the eggs were first placed in the cyanide solution; *i.e.*, most were approximated and had embraced the vessels, but a few, especially those of the red variety, were still partially spread out over the surface of the yolk sac. At this time the controls, which had been kept in sea water without cyanide, had hatched. Those placed in the cyanide never hatched. When kept for 9 days in 50 cc. of sea water plus two drops of sodium cyanide the circulations were inactive but the hearts gave occasional spasmodic beats or beat steadily. The chromatophores had not spread out as they did when the circulation was stopped before the heart in Experiments 1 and 2. After removal from the cyanide to sea water or potassium chloride solutions the chromatophores behave exactly as they did under similar conditions in Experiments 1 and 2. The cyanide by lowering the rate of oxidation may have interfered with the motility of the chromatophores.

Experiments with alcohol were next tried.

Eggs fertilized 4 days previously were placed in sea water to which absolute ethyl alcohol had been added in sufficient quantity to make solutions of 2, 3, 4, and 5 per cent respectively. Thirty egg were placed in each solution. After 18 hours ten eggs were removed from each solution and placed in sea water. The same procedure was repeated after 24 and again after 30 hours and each lot of eggs, separately kept, was observed at frequent intervals. Table II gives the results.

TABLE II.

Day of incubation.	2 per cent.			3 per cent.			4 per cent.			5 per cent.			Control.
	18 hrs.	24 hrs.	30 hrs.	18 hrs.	24 hrs.	30 hrs.	18 hrs.	24 hrs.	30 hrs.	18 hrs.	24 hrs.	30 hrs.	
5	++	++	++	++	++	++	++	+	+	0	0	0	++
6	++	++	++	++	++	++	++	+	+	+	0	0	++
10	++	++	++	++	++	++	++	++	+	++	+	0	++
20	H.	H.	H.	H.	H.	H.	H.	H.	H.	H.	+	D.	H.
30											+		

++, hearts and circulations active.

+, hearts but not circulations active.

0, neither hearts nor circulations active.

H., hatched.

D., dead.

In Table II majorities are indicated as the results were too irregular to record *in toto* without too much complexity. However, the results in each instance are similar to those encountered in previous experiments in every particular. Where the circulation is active (++), the chromatophores closely approximate the blood vessels and tightly embrace them. The embracing process is less complete the shorter the time since the circulation has again commenced; *i.e.*, 5 per cent, 10th day of incubation. When the heart is active but the circulation is at a standstill (+), the chromatophores are widely spread out over the yolk sac; *i.e.*, 5 per cent, 6th day of incubation. This is also the case when neither the heart nor the circulation is active (0); *i.e.*, 5 per cent, 5th day of incubation. There was considerable mortality among the embryos treated with alcohol, and in certain instances the circulations in some of the embryos were inactive while others were active. The tendency is indicated by the graphic record of the majority. When variations occurred there was always the consistent relation between the circulation and the chromatophores.

DISCUSSION.

The distribution and arrangement of the chromatophores of the yolk sac of embryonic *Fundulus heteroclitus* seem to be determined by their relation to the most abundant supply of oxygen or to the lower hydrogen ion concentration. Thus, before there is any circulation they are spread out widely on the surface of the yolk sac without regard to the blood vessels. As soon as the circulation starts, the chromatophores approach the capillary, creeping longitudinally along the surface of, coupling up with those adjacent, and finally, forming an almost continuous covering of the vessels of the yolk sac. It may be presumed that at this time the tension of oxygen is higher and the hydrogen ion concentration lower in the circulating blood than in the tissue cells. If the circulation is stopped by any means, potassium chloride, sodium cyanide, ethyl alcohol, even with a beating heart, the chromatophores tend to spread out once more over the surface of the yolk sac in such a way that the greatest possible surface area is approximated to the sea water surrounding the embryo. With a beating heart, however, a clump of chromatophores is often found about the base of this organ, for reasons not entirely clear. In the experiments recorded above all stages in the development of a circulation and the cessation of one once begun have been encountered and it may be safely stated that there seems to be a direct relation between an active circulation and a close approximation and envelopment of the vessels by the chromatophores. A substance which, like sodium cyanide, inhibits oxidation also inhibits further migrations of the chromatophores, probably by paralyzing the latter; the tropistic reactions of the chromatophores are resumed when the poison (sodium cyanide) is removed and the eggs are transferred to a proper medium (sea water). When the eggs die the chromatophores shrivel up and appear as small dark spots against the opaque background of the egg. The red chromatophores respond much more slowly to an adequate stimulus than the black ones.

CONCLUSIONS.

1. The migrations of the chromatophores of the yolk sac of *Fundulus heteroclitus* seem to be chemotropic in character.

2. The determining factor seems to be either a high tension of oxygen or a comparatively low concentration of hydrogen ions.

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NATURE OF THE RETARDING INFLUENCE OF THE THYMUS UPON AMPHIBIAN METAMORPHOSIS.

BY EDUARD UHLENHUTH.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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There is a general agreement concerning the influence of the thyroid upon the metamorphosis of anuran amphibian larvæ. All authors who reported experiments on feeding thyroid to tadpoles, regardless of the locality in which they were carried on and on whatever species of anura they were performed, invariably found that thyroid feeding always resulted in a precocious metamorphosis. The experiments on thymus feeding, however, are characterized by just the opposite feature, the results being very inconstant. Thymus feeding sometimes resulted in retardation or entire prevention of metamorphosis; sometimes it had no effect as compared with the controls. Such different action not only was obtained in the experiments of different authors, performed on different species and at different places (Gudernatsch obtaining positive, Swingle negative results), but even when the same investigator experimented on the same species different individuals were affected differently by the thymus diet (Romeis). Nevertheless, it is certain that in some cases thymus feeding actually prevented metamorphosis and resulted in giant larvæ, while no such effect was obtained in the controls on the normal diet.

It seems necessary therefore to explain why thymus feeding sometimes does and sometimes does not prevent metamorphosis.

The writer performed a large number of experiments on the larvæ of *Ambystoma maculatum*,[1] *Ambystoma opacum*, and *Ambystoma tigrinum*, and it was found that while the thyroid contains a specific substance enforcing metamorphosis, the preventive effect of thymus feeding is due to the absence from the thymus of a substance necessary for the formation or excretion by the thyroid of the substance causing metamorphosis.

[1] For nomenclature of salamanders see Stejneger, L., and Barbour, T., A check list of North American amphibians and reptiles, Cambridge, 1917.

Inhibitory Effect of the Thymus.

Some experiments may be reported briefly showing that thymus feeding sometimes actually prevents metamorphosis, though in the majority of the larvæ it has no such effect.

In a set of larvæ of *Ambystoma maculatum*, kept at approximately 15°C., fed on tubifex and earthworms, and consisting of about 100 specimens, most of the larvæ, which had hatched in the beginning of May, 1916, metamorphosed during the months of August and September. On October 21, eight of these were still larvæ. These were picked out to form a series of normal controls (c. W. 1916). In a set of eighteen larvæ of the same species and of the same age and kept under the same conditions as the above series, but fed on thymus soon after hatching, nine larvæ had metamorphosed up to October 21. The remaining nine were picked out to form a thymus-fed series (c. T. 1916). The worm-fed larvæ of Series c. W. 1916 metamorphosed at an age of 28 weeks and 6 days on an average; the first larva to metamorphose was 25 weeks and 4 days old at the time; the last one 33 weeks and 4 days. Among four larvæ of the thymus-fed Series c. T. 1916, the first animal was 27 weeks old when it metamorphosed, the last one 55 weeks and 2 days. Of these four larvæ the average age at the time of metamorphosis was 35 weeks and 2 days. The other five larvæ of the thymus-fed series died before metamorphosis; three at the age of 31 weeks and 2 days, one at the age of 40 weeks and 3 days, and one at the age of 62 weeks and 3 days. Thus one animal of the thymus-fed series remained larval for about 1 year and 3 months; at this time it did not show any signs of metamorphosis and it seems possible that it would have remained permanently in a larval state. The writer so far has never observed in his normal controls individuals which remained larval for so long a time. Thus there can be no doubt that in this thymus-fed series metamorphosis was retarded as compared with the controls and in one case probably was even prevented.

In a series of eight larvæ of *Ambystoma opacum*, which were kept at about 25°C. and fed moderately on earthworms, the average time of metamorphosis was 26 weeks; in a series of eight larvæ of the same species of the same age and from eggs of the same female as the

larvæ of the foregoing series, the larvæ were fed on thymus and with the largest possible amount of this diet; otherwise they were kept like the worm-fed controls. The average time of metamorphosis in this series was 17 weeks and 5 days; *i.e.*, less than in the worm-fed series. In this number, however, two larvæ are not included, both of which died before metamorphosis; one of them reached the age of 31 weeks and 2 days, the other 34 weeks and 1 day without having metamorphosed. Thus in this series again a considerable delay (if not an inhibition) of metamorphosis was produced by the thymus diet in two animals.

A third species, *Ambystoma tigrinum*, was experimented on. This species, as mentioned in a recent publication[2] shows the least effects when fed on thymus. Among six animals kept at approximately 25°C. and fed on thymus, the larvæ metamorphosed simultaneously with the controls. Among six other animals kept at approximately 15°C. and fed on thymus, only five larvæ metamorphosed simultaneously with the controls (between 22 and 26 weeks after hatching), while one individual though now over 74 weeks old is still in larval condition. Thus also in this species thymus feeding apparently resulted in a considerable retardation of metamorphosis though only in one individual.

From these experiments on urodelan larvæ it is again evident that the effect of the thymus is extremely variable. But it is also a fact that in some of the larvæ metamorphosis was retarded by the thymus diet and in two probably completely prevented. Further experiments, therefore, were carried out to determine this point.

Inhibitive Effect of Thymus upon Amphibian Metamorphosis Is a Deficiency Phenomenon.

It is clear that the considerable variability of the action of the thymus cannot be explained on the assumption that the inhibitive effect of that gland is due to the presence of a specific inhibiting substance in the thymus. But it can be explained if this effect is due to the absence from certain parts of the thymus of a substance necessary to produce metamorphosis and which is contained, in minute quan-

[2] Uhlenhuth, E., *J. Gen. Physiol.*, **1918, i, 23.**

tities, in other parts of the thymus, in normal food, and perhaps in the water of certain localities where unsuccessful experiments on thymus feeding have been carried out.

If the preventive influence, which the thymus exhibits in some of the larvæ, is due to the presence of some specific metamorphosis-inhibiting substance, metamorphosis evidently should be prevented by the thymus even if normal food is added to the thymus diet. This is the case, for instance, with the metamorphosis-producing substance of the thyroid gland. Lenhart[3] has shown that if a certain amount of the active substance of the thyroid, able to produce accelerated differentiation and not large enough to result in death from emaciation before differentiation can take place, is introduced into the organism, differentiation will be accelerated and at a definite rate, whether the tadpoles are fed only on thyroid or whether some other food (liver) is added to the glandular diet. In fact, it seems, from all experiments so far performed with thyroid, that it is of no importance what food the larvæ receive; the addition of only a minute quantity of thyroid substance causes metamorphosis at an accelerated rate. We found the same to be true for the larvæ of salamanders. Young (5 weeks old) larvæ of *Ambystoma opacum* which were fed on earthworms, were placed in a 0.02 per cent solution of iodothyrin; in spite of the earthworm diet and of the small quantity of thyroid substance used (Bayer's iodothyrin), all larvæ metamorphosed from 8 to 9 days after the commencement of the thyroid treatment, while the controls needed 7 to 8 weeks more to metamorphose. The thymus itself contains a specific substance which is highly toxic and produces tetany in the larvæ of *Ambystoma maculatum* and *opacum* as described in a former article.[2] The action of this substance as regards its constancy is quite similar to the action of the metamorphosis-producing substance of the thyroid and very unlike the metamorphosis-inhibiting action of the thymus. No matter what food is added to the thymus diet larvæ fed on thymus always had tetany. And like the thyroid substance, the tetany toxin of the thymus is also characterized by the constancy of its action; it produces tetany in each individual.

[3] Lenhart, C. H., *J. Exp. Med.*, 1915, xxii, 739.

It is quite different with the prevention of metamorphosis by the thymus. If normal food is added to the thymus diet, metamorphosis will take place in each individual at the same time as in the controls. This is shown in the following experiments on larvæ of *Ambystoma maculatum* of the same age, from eggs of the same female, and all kept at approximately 25°C. One series of 30 larvæ was fed only on worms, one series of 15 larvæ only on thymus, and one series on thymus and worms alternately. Fig. 1 shows the result. The larvæ

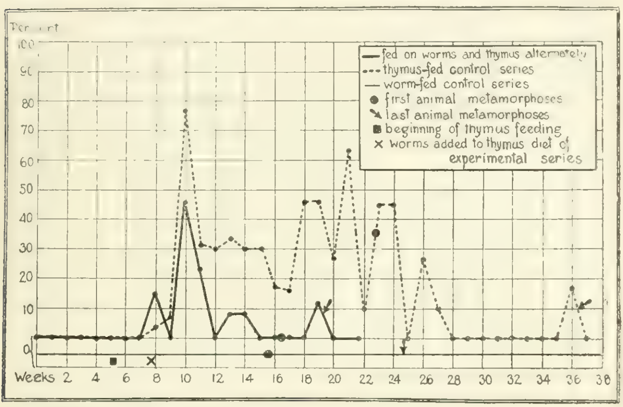

FIG. 1. Effect of worm diet when added to thymus diet upon metamorphosis and tetany in *Ambystoma maculatum* larvæ. Curves indicate percentage of tetanic animals.

of the mixed food series metamorphosed simultaneously with those of the control series, which were fed on worms only; while in the series fed on thymus, metamorphosis started when all animals of the series fed on worms and on worms and thymus alternately had already metamorphosed, and the last larval animal died about 12 weeks later without having metamorphosed at all. In other words, in the series fed on mixed diet the food added to the thymus diet contained the substance which is necessary for metamorphosis and which is lacking in the thymus; and thus this substance was introduced into

the larval organism in such a large amount that metamorphosis occurred in this series simultaneously with the controls. The ability of the normal food to counteract the inhibitive effect of the thymus upon metamorphosis was the more conspicuous as the same food was quite ineffective in preventing the tetanic convulsions produced by the tetany toxin present in the thymus; this may be seen from the curves which indicate the percentage of tetanic animals found at the time of observation among the thymus-fed and mixed food series.[4]

That the action of the thymus is merely due to the absence of the substance necessary to produce metamorphosis, is also demonstrated in experiments in which thymus-fed larvæ are placed in a solution of thyroid substance. If the thymus contained a specific metamorphosis-preventing substance, one would expect an antagonistic neutralization of the thyroid substance by the thymus substance. But instead the thyroid substance even when present in minute quantities induces prompt metamorphosis in the thymus-fed animals. For the sake of illustration one experiment may be reported here. A set of six thymus-fed larvæ of *Ambystoma opacum* was fed on thymus. At an age of 6 weeks the larvæ were placed in a 0.02 per cent solution of iodothyrin which after about 20 hours was replaced by a 0.006 per cent solution. 8 days after the beginning of the thyroid treatment all the larvæ were metamorphosed although fed on thymus, while the controls not treated with iodothyrin needed from 6 to 7 weeks more to metamorphose. In this series again we observe that the effects of the thymus, which actually are due to the presence of a special[5] substance in the thymus, are not stopped by the thyroid treatment. The larvæ exhibited severe tetanic convulsions caused by the tetany toxin of the thymus; these convulsions occurred in spite of the thyroid treatment with undiminished strength.

[4] For detailed discussion see Uhlenhuth, *J. Gen. Physiol.*, 1918, i, 33.

[5] The term specific in connection with the active principle of the thyroid gland has been avoided here, for it does not seem to be proved definitely that the effects exerted by the iodothyrin cannot be brought about by any other substance or any other factor. Since the term specific refers not only to the origin of the inner secretory substances but also to their effects, it is misleading in connection with the thyroid substance.

DISCUSSION.

From the above experiments it is evident that an exclusive thymus diet sometimes can retard or even prevent metamorphosis. But while the ability of the thyroid to enforce metamorphosis is due to the presence in the thyroid of a special substance, the inhibitory action of the thymus is due to the absence of a substance without which metamorphosis is impossible.

In order to appreciate this fact fully we must remember the experiments performed by Allen[6] and Hoskins,[7] which have demonstrated that tadpoles whose thyroid glands have been extirpated are unable to metamorphose. This means that under normal conditions at the time of metamorphosis the thyroid begins to excrete the metamorphosis-producing substance which under experimental conditions is introduced into the organism by feeding thyroid to the larvæ or keeping them in a solution of thyroid substance. Since under normal conditions no thyroid is fed to the animals and since it is the thyroid of the animal itself which excretes the substance in question, we must assume that the normal food of the larvæ contains a substance which is necessary to develop the thyroid of the larva to a state in which it can excrete the metamorphosis-producing substance. Whether or not the substance necessary to develop the thyroid and furnished in normal conditions by the normal food of the larvæ is identical with the metamorphosis-producing substance excreted later on by the thyroid, cannot be decided at present; but in this respect the attempts made by Allen[6] to enforce metamorphosis of thyroidless larvæ by feeding them on thyroid are important. If it is possible to enforce metamorphosis in thyroidless larvæ by feeding them on thyroid, but impossible by feeding them normal food, the substances contained in the normal food are able to develop the thyroid to the excreting stage, but they are unable to evoke metamorphosis in the absence of the thyroid, and, therefore, they are not identical with the thyroid substance. The results so far obtained by Allen point in the latter direction. It is this substance, necessary to develop the secretory stage in the thyroid, which is missing in the thymus.

[6] Allen, B. M., *Science*, 1916, xliv, 755; *J. Exp. Zool.*, 1917–18, xxiv, 499.

[7] Hoskins, E. R., and Hoskins, M. M., *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 102.

The question arises now in which way do some of the thymus-fed larvæ procure enough of the substance required for the development of the thyroid, while other thymus-fed larvæ are unable to obtain enough of it though all of these larvæ apparently are fed on an equally exclusive thymus diet? Although at present an exact statement on this matter is impossible we must seek its explanation in the fact that evidently the amount required is so small that it was difficult in our experiments so far performed to control the sources which sometimes supplied this substance against our will. As to the actual source of the substance it is possible that the connective tissue constituting the septa between the lobules of the thymus may contain some of it; in fact, this is probable since the septa are in no way a specific tissue like the rest of the thymus. The possibility of some of the larvæ having obtained, by chance, more of the septa than others must be admitted. In this way the great variability of the results of the same author could be explained. There is also a possibility that the water may contain some of that substance; in favor of this would be the fact that some authors, like Swingle,[8] did not obtain any retarding effects at all in their thymus-fed series. Of course, the difference in the reaction of different individuals of the same series of one experimenter can be explained less readily on that possibility.

Our experiments suggest the possible character of the influence of environmental factors on metamorphosis. The problem of amphibian metamorphosis, as well as the problem of internal secretion, assumes a new shape in the light of that fact. On the one hand, it has become clear from the experiments of Allen and his followers that metamorphosis is directly dependent on the action of a certain inner secretory gland of the amphibian larva; on the other hand, it is evident that the development of the secretory stage of that inner secretory gland depends ultimately on certain purely environmental, non-glandular factors. And it is now time that we should recall such attempts as those made by Duméril[9] and von Chauvin[10] to enforce or prevent metamorphosis by purely external non-glandular

[8] Swingle, W. W., *J. Exp. Zool.*, 1917–18, xxiv, 521.
[9] Duméril, A., *Ann. sc. nat., Zool.*, 1867, vii, 229.
[10] von Chauvin, M., *Z. wiss. Zool.*, 1885, xli, 365.

factors; these attempts in the case of von Chauvin doubtless were successful, though the actual relation between the factors employed and the result obtained cannot well be understood at present.

SUMMARY.

1. Though thymus-fed salamander larvæ often metamorphose normally, thymus feeding sometimes retards and in rare cases inhibits metamorphosis completely.

2. The addition of normal food to a thymus diet abolishes the inhibitory effect of the thymus.

3. Addition of a small quantity of iodothyrin leads rapidly to precocious metamorphosis of thymus-fed larvæ.

4. The inhibitory effect of the thymus is not due to a specific inhibiting substance in the thymus, but to the absence from the thymus of a substance required to develop the thyroid to the secretory state.

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PARATHYROIDS AND CALCIUM METABOLISM.

By EDUARD UHLENHUTH.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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MacCallum and Voegtlin,[1] as well as other authors, have found that in tetany resulting from the extirpation of the parathyroids the Ca content of the blood and the organs (brain) is greatly reduced and that the introduction into the organism of Ca salts, subcutaneously, intravenously, or *per os*, suppresses the tetanic convulsions of the animals operated on. These findings have been confirmed recently by Howland and Marriott[2] in tetany of children. Spontaneous tetany in human beings has apparently the same cause as parathyreoprival tetany, both being due to the non-functioning of the parathyroids. Furthermore, it is known[1] that after parathyroidectomy, tetanic convulsions may be suppressed by bleeding the animals and substituting the amount of blood drawn by an equal amount of salt solution. From the latter fact MacCallum and Voegtlin conclude that in the absence of the parathyroids some toxic substance accumulates in the blood, which normally is antagonized by the parathyroids. They assume further that the toxicity of this substance is due to its ability to combine, in some unknown way, with calcium which it extracts from the organs, causing its excretion and thereby diminishing the Ca content of the blood and organs. MacCallum thinks that the muscular convulsions in tetany are the result of the diminution of the Ca concentration, the function of the parathyroids being to regulate the Ca concentration by antagonizing the toxic substance and thus preventing it from extracting the Ca salts from the body.

As regards the existence of a toxic substance involved in the causation of tetany, the writer has shown that such a substance actually

[1] MacCallum, W. G., and Voegtlin, C., *J. Exp. Med.*, 1909, xi, 118.

[2] Howland, J., and Marriott, W. McK., *Bull. Johns Hopkins Hosp.*, 1918, xxix, 235.

exists and is contained in the thymus gland.[3] In the present article certain experiments will be reported demonstrating that calcium is able to suppress the tetanic convulsions, at least to some extent; the writer, however, was unable to convince himself that this effect upon tetany is characteristic for the calcium and furthermore the experiments in question, though they do not exclude a possible relation between the toxic substance and the calcium, prove conclusively that, as far as the animals used in these experiments are concerned, the tetany toxin, even in the presence of the calcium and in the absence of convulsions, brings about severe lesions of the muscular system resulting probably from lesions of the central nervous system caused by the tetany toxin and not prevented by the calcium.

EXPERIMENTAL.

In order to test the action of Ca lactate upon tetanic animals, a number of larvæ of the salamander *Ambystoma opacum* were fed on thymus and kept at the same time in a solution of Ca lactate in ordinary tap water; another set of larvæ of the same age and from the same female were kept in a solution of Mg lactate of the same concentration as the Ca lactate solution. A series of larvæ from a different female, fed on thymus, but kept in ordinary tap water, served as controls; since differences between larvæ from different females as regards the severity of tetany when fed on thymus are so small as to be negligible, the error introduced by comparing larvæ of different females is very small. For each of the three thymus series one series was kept as control, in which all conditions were the same as in the thymus-fed series, except that small pieces of earthworms instead of thymus served as food. None of the worm-fed control series developed tetany.

I. Thymus-Fed, Untreated Controls (Fig. 1, Curve I).—Six larvæ of *Ambystoma opacum* were fed on thymus exclusively. As usual the tetanic attacks began after the larvæ had reached a certain developmental stage[3] and soon reached a maximum. Each single individual came down with tetanic convulsions. When metamorphosis was

[3] Uhlenhuth, E., *J. Gen. Physiol.*, 1918, i, 23, 33.

approached, tetanic convulsions ceased, and after metamorphosis all larvæ were free from it.

Besides the tetanic convulsions the other symptoms of tetany were also present in all animals; the legs and feet became permanently twisted and contracted and the entire body assumed the shape char-

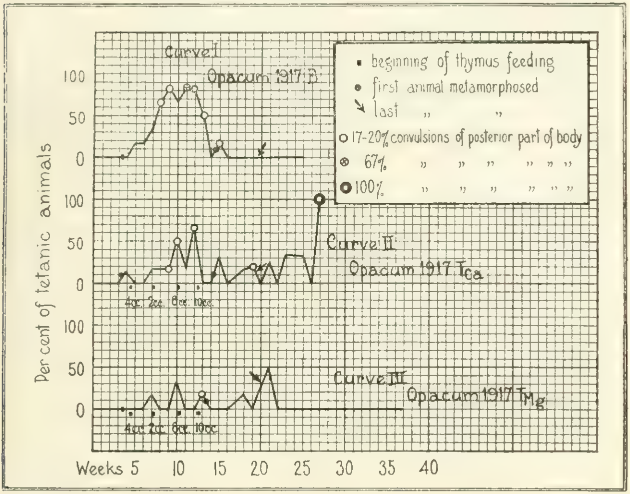

FIG. 1. Tetanic convulsions in a thymus-fed series of *A. opacum* (Curve I); in a thymus-fed series, kept in Ca lactate (Curve II); and in a thymus-fed series, kept in Mg lactate (Curve III). The ordinates indicate the percentage of animals showing tetanic convulsions at the time indicated on the abscissæ. Convulsions of the entire body and convulsions of the posterior portion of the body alone are considered.

acteristic of tetanic animals. The limbs and most of the muscles necessary for the movements of the animals became permanently paralyzed.

II. Thymus-Fed Series, Kept in Ca Lactate (Fig. 1, Curve II).— Seven larvæ of *Ambystoma opacum* were used, one of which died soon after the experiment had been started. Several days after the thy-

mus feeding had been started, one of the larvæ had a tetanic attack. 8 days after the beginning of thymus feeding the larvæ were transferred to a 1/1250 M solution of Ca lactate in tap water; this suppressed tetanic convulsions up to the 7th week, while in the untreated controls tetany had started at the end of the 5th week. Though the concentration of the solution was lowered to only 1/2500 M Ca lactate, the tetany curve did not rise as it did in the controls, but remained low until at the end of the 10th week. The concentration of the solution was increased to 1/625 M, upon which the curve fell, but soon it rose again and, after a further increase of the concentration to 1/500 M, it fell to zero; this latter fall, however, is probably due in part to the approach of metamorphosis, though the rise of the curve soon after the commencement of metamorphosis was due to tetanic convulsions of two larvæ.

As compared with the control thymus series the tetanic convulsions in the Ca lactate series were doubtless somewhat decreased; in particular it is very evident that an increase of the concentration resulted in a fall of the curve. It is worth noting that in the Ca series as in the untreated thymus series each single individual suffered from tetanic convulsions though for a shorter period of its larval life than the larvæ of the thymus-fed, control series.

The most important observation, however, is that though the muscular convulsions were decreased by the action of the Ca salt, the other symptoms of tetany, in particular the permanent paralysis of almost the entire muscular system, developed at the same time and with the same severity as in the untreated thymus-fed control series.

Finally, it should be mentioned that in the Ca series the metamorphosed animals behaved entirely differently from untreated thymus-fed animals. When the larvæ metamorphosed, they were taken out of the solution and placed on moist filter paper. As in all other thymus-fed animals, their muscles were paralyzed and the shape of the body and of the legs was greatly deformed, abnormalities which they had acquired during the tetanic period; like the untreated thymus-fed animals, they did not suffer from tetanic convulsions. Several weeks, however, after metamorphosis tetanic convulsions started again in the Ca animals, in contradiction to what we have observed

in all the untreated thymus-fed animals. This surprising difference has not found so far any explanation and will not be considered in the present article, detailed discussion being reserved until further experiments upon this phenomenon are available.

III. Thymus-Fed Series, Kept in Mg Lactate (Fig. 1, Curve III). – Seven larvæ of *Ambystoma opacum* were used; one of them died soon after the experiment had been started. 8 days after the beginning of the thymus feeding and before tetanic convulsions had made their appearance, the larvæ were transferred to a 1/1250 M solution of Mg lactate in tap water. By this concentration tetanic convulsions were suppressed until the end of the 7th week; at this time one larva had convulsions. But even though the concentration had been lowered to only 1/2500 M, no further convulsions occurred until the 10th week; the concentration was increased to 1/625 M, upon which the curve fell immediately to zero. The concentration was further increased to 1/500 M; only one larva developed tetanic convulsions of the posterior portion of the body, and no further attacks were observed among the larvæ, the rise of the curve at the end of the 18th week being due to tetany of a metamorphosed animal.

In this series the Mg lactate had a distinct and very definite influence upon the frequency and severity of the muscular convulsions; not only is the curve running far lower than in the untreated thymus-fed series, but it is also lower than in the Ca series. Furthermore, three of six larvæ had no muscular convulsions during the larval period. Evidently the effect of the Mg lactate in suppressing the muscular convulsions during tetany is far greater than that of the Ca lactate, when used in the concentrations employed in these experiments.

This influence of the Mg lactate upon the convulsions of the muscles, however, does not mean that Mg is able to suppress tetany, for the other symptoms of tetany, *i.e.* paralysis of the muscles and deformation of the extremities and of the body, develop and to the same degree as in non-treated thymus-fed larvæ.

Concerning the metamorphosed animals the same phenomenon is observed as in the Ca series. A number of weeks after the animals had metamorphosed and were set on moist filter paper without being exposed longer to Mg lactate, they began to suffer again from tetanic

convulsions. And two of the animals which did not have convulsions during their larval period had convulsions after metamorphosis. But the third specimen, which had no convulsions during its larval life, never had tetanic convulsions; it was still alive 43 weeks after metamorphosis.

DISCUSSION AND CONCLUSIONS.

The experiments reported in this article are in full agreement with the facts known about the action of Ca and Mg salts in tetanic animals. In the concentrations used here both Ca lactate and Mg lactate suppressed the muscular convulsions in the tetanic salamander larvæ. The Mg lactate, however, appears to be more effective than the Ca lactate. At any rate the suppression of the tetanic convulsions does not seem to be a specific action of the calcium.

The most important result seems to be the fact that the salts used, though they prevented the muscular convulsions, did not prevent the other symptoms of tetany which in the salamander larvæ are very definite and constant. The permanent spasmodic contractions and the paralysis of the muscles developed in spite of the presence of the Ca and Mg. Furthermore, the muscular contractions and the paralysis developed even in such thymus-fed animals in which the convulsions had been suppressed completely; this was the case in one of the animals of the Mg series.

From the experiments of Biedl[4] and others it is likely that the tetanic convulsions are due to lesions of the central nervous system, since convulsions of a leg can be prevented by isolating it from the central nervous system by cutting the nerves which connect the muscles with the central nervous system. Evidently these lesions of the central nervous system are the chief factor in tetany, while the convulsions of the muscles are only an effect. In the larvæ of salamanders these lesions find a definite expression in the permanent paralysis of almost the entire muscular system.

In the writer's opinion, MacCallum's hypothesis that the tetany toxin has a special affinity for Ca, thereby diminishing the Ca content of the organism, cannot be disproved at present. But the

[4] Biedl, A., Innere Sekretion, Berlin and Vienna, 1913, i, 126.

present experiments seem to prove, first, that the tetany-producing substance causes permanent lesions of the nervous system, which lead to permanent spasmodic contractions and paralysis of the muscle even in the absence of tetanic convulsions, and second, that these cannot be prevented by either Ca or Mg. For the most part they result in an early death of the animals no matter whether or not Ca or Mg has been applied.

In connection with this fact we wish to mention Biedl's claim[4] that no one has yet succeeded in prolonging the life of parathyroidectomized animals by the application of Ca. From MacCallum's paper, on account of the lack of controls, it cannot be seen whether his parathyroidectomized dogs lived longer with Ca treatment than without.

That in spontaneous tetany Ca treatment may effect a cure, as is evident from the report by Howland and Marriott, does not prove that in this case Ca has inhibited tetany as a disease. In spontaneous tetany the period of the action of the tetany-producing substance may be a very short one and the mere prevention of the tetanic convulsions may keep the patient alive until normal function of the glands involved has been restored. The pathological changes which the central nervous system undergoes in this short period may not be severe enough to endanger the life of the patient after the cessation of the action of the tetany toxin.

In the light of the facts presented our experiments lead to the following conclusions:

1. The thymus gland excretes a tetany-producing substance which in the normal animal is antagonized in an unknown way by the parathyroids.

2. In animals devoid of parathyroids (salamander larvæ, parathyroidectomized mammals) this substance may, according to MacCallum, reduce the Ca content of the organism; but by far the most dangerous and important quality of this substance is its highly injurious effect upon the central nervous system, which causes permanent spasmodic contractions of the muscles and paralysis of almost the entire muscular system.

3. It is possible to prevent the muscular contractions by introducing Ca salts into the body, though this can be done more effectively by means of Mg salts.

4. No substance, however, has been found so far to antagonize the tetany toxin and to prevent the development of the lesions of the central nervous system caused by the tetany toxin.

5. This explains why in spite of the application of Ca or Mg and in spite of the suppression by these substances of the tetanic convulsions the other symptoms of tetany develop and frequently lead to the death of the animal.

6. Accordingly the most important function of the parathyroids is to prevent the tetany toxin, by antagonizing it, from coming into contact with the central nervous system.

The writer wishes to express his thanks to Dr. G. M. Meyer, of The Rockefeller Institute, for preparing the solutions used in these experiments.

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NON-LACTOSE FERMENTING BACTERIA FROM POLLUTED WELLS AND SUB-SOIL.*

BY I. J. KLIGER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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While *B. coli* is generally accepted as a satisfactory index of pollution, it is aways desirable to detect the presence of specific pathogens in any suspected material. The methods for isolating such pathogens, though highly developed, are far from satisfactory. In water, one encounters the problem of high dilution, while in contaminated soil, the presence of large numbers of organisms of the *B. proteus* group interferes greatly with the successful detection of typhoid, dysentery, and related types of bacilli. The failure to find the latter organisms does not, therefore, imply their absence, though it may indicate that they are not present in large numbers.

Methods.

The samples of soil and water were collected in sterile wide mouth, glass stoppered bottles. The soil specimens were removed to sterile Petri dishes, thoroughly mixed, and portions of 10 to 25 grams weighed into sterile glass stoppered bottles. Sterile water was then added in the proportion of 2 cc. of water to 1 gram of soil, the bottles were shaken vigorously for a few minutes and then allowed to stand until the heavy particles had settled to the bottom. The supernatant suspension was then tested by two or all of the following methods:

Direct Plate Method.—The water and soil suspensions were plated direct on Endo and brilliant green plates, 0.1 cc. being spread on the former, and 0.2 cc. on the latter, respectively. The plates were incubated for twenty-four hours and suspicious colonies fished to Russell

* Work conducted under a grant of the International Health Board of The Rockefeller Foundation, New York.

double sugar tubes. All the Gram negative non-lactose fermenting bacilli were kept for further study.

Bile Enrichment Method.—Portions of 10, 2, and 0.2 cc. of the soil suspension and 10, 1.0, and 0.1 cc. of the water were inoculated into lactose bile and the tubes incubated for forty-eight to seventy-two hours. A loopful of fluid from all the tubes that showed gas was then spread on both brilliant green and Endo plates, and the suspicious colonies appearing after incubation for twenty-four hours were inoculated into Russell double sugar tubes, and treated as above.

Agglutination with Polyvalent Anticoli Serum.—This method was the converse of that used by Adami and Chopin (1904). Adami and Chopin reported successful results in the isolation of *B. typhi* from water with the aid of antityphoid agglutinating serum. Preliminary tests of this procedure gave unsatisfactory results, because *B. coli* invariably agglutinated together with the *B. typhi* in sufficiently large numbers to crowd out the latter on plates. It seemed that a polyvalent colon agglutinating serum would be more generally applicable, in that it would remove the *B. coli* and leave the specific pathogens, as well as the other non-lactose fermenting bacilli, in the supernatant fluid.

By inoculating 5 different varieties of *B. coli* into a sheep, a serum was obtained that could agglutinate 50 to 60 per cent of a heterogeneous collection of 50 strains of *B. coli*. No agglutinating serum could be obtained against *B. aerogenes* and a type of *B. coli-communior*, which is apparently very close to the capsulated *B. aerogenes*.

This anticolon agglutinating serum was used successfully in the isolation of non-lactose fermenting bacilli from a few samples of soil, water and feces. The procedure was as follows: The serum was added to beef bouillon or peptone broth in a concentration of 1: 100. Then 10 cc. of water or the soil suspension or a loop of feces were added to 10 cc. of broth, and the tubes incubated until flocculation was visible. (The period of incubation depended, of course, on the number of *B. coli* present at the time of incubation and varied from two to six hours.) The tubes were then centrifuged at low speed for 5 minutes, and the supernatant fluid plated on Endo and brilliant green plates. Two samples of soil, 2 of water, and 2 stools from patients

suffering with summer diarrhea yielded successful results with this method. From the stools practically pure cultures of *B. dysenteriæ* (Flexner) and the Morgan bacillus respectively were obtained with this procedure.

Results.

By the use of the method outlined above, either singly or in combination, non-lactose fermenting, non-liquefying bacteria were obtained from 15 polluted wells and 14 contaminated sub-soil samples. In but one instance were members of this group obtained from both the well and soil of the same premises, but even in this case the organisms possessed different biological and serological characters.

The predominant organism obtained from the wells differed from the one found in the sub-soil. The fifteen wells yielded 3 organisms resembling the acid type of *B. dysenteriæ*, 2 strains of the Morgan bacillus no. 1, 9 para-enteritidis, and 2 paracolon bacilli. The soil samples, on the other hand, gave 9 dysentery-like bacillary strains, 7 of which corresponded culturally with the acid and 1 with the Shiga type; 4 para-enteritidis, and 2 paracolon bacillary strains. In only two instances were non-lactose fermenting bacilli obtained from sub-soil at a distance greater than 2 feet from a privy pit. Both were sandy soils examined during the wet period. The distance from the privy in these two cases was 10 feet and the depths were $7\frac{1}{2}$ to $8\frac{1}{2}$ feet respectively.

It is noteworthy that the prevalent type in wells belongs to the paratyphoid or para-enteritidis bacterial group, while that found in polluted soil resembles the dysentery bacillus. Smith and Moore (1893) found members of the paratyphoid group in the feces of normal cattle, swine, and other domestic animals. Savage (1906–1910) carried out an extensive investigation on the prevalence of non-lactose fermenting bacilli in excreta of man and animals. He reports the presence of para-Gärtner bacilli in large numbers in normal swine, calves, horses, and mice; but claims that in man they are encountered only infrequently and in small numbers. The true Gärtner bacillus was detected by him once only (in human excreta); the others were what he terms the para-Gärtner bacilli, which dif-

TABLE I.

The Fermentation and Agglutination Reactions of the Non-Lactose Fermenting Bacilli Isolated from Polluted Wells and Soil.

NUMBER	SOURCE	FERMENTATION REACTION									AGGLUTINATING SERA				TYPE RESEMBLED
		Glucose	Mannite	Sucrose	Xylose	Arabinose	Rhamnose	Salicin	Dulcite	Indol	Enteritidis	Paratyphi B	Hog cholera	Dysentery	
155	Soil	+	+	–	–	+	–	–	–	–				–P	Flexner dysentery
493	Soil	+	+	–	–	+	–	–	–	±				+(100)F	Flexner dysentery
395	Well	+	+	–	–	+	–	–	–	–				+(100)F	Flexner dysentery
112	Soil	+	+	–	–	–	–	–	–	–				–P	
384	Soil	+	+	–	–	–	–	–	–	–				–P	
152	Soil	+	–	–	–	–	–	–	–	–				–P	Shiga (not toxic)
75	Soil	+	+	+	+	+	–	–	–	–				±(500)F	Strong dysentery
84	Soil	+	+	+	+	+	–	–	–	–				+(100)F	Strong dysentery
512	Soil	+	+	+	+	+	–	–	–	–				+(1000)P	Strong dysentery
437	Well	+	+	+	+	–	–	–	–	–				–P	?
388	Well	+	+	+	+	–	+	–	–	–				±(1000)P	?
60	Soil	+	+	+	+	+	+	–	–	+				±(500)F	?
57 b	Well	*	–	–	–	–	–	–	–	+	+1000	–	–	–	Morgan no. 1
318	Well	*	–	–	–	–	–	–	–	+	+1000	–	–	–	Morgan no. 1
286	Well	*	*	–	*	*	*	–	–	–	++100	–	–	–	Enteritidis
30	Well	*	*	–	*	*	*	–	–	–	±100	–	–		Enteritidis
33	Well	*	*	–	*	*	*	–	–	–		–	–		Enteritidis
46	Well	*	*	–	*	*	*	–	–	–	+100	±100	–		Enteritidis
57a	Well	*	*	–	*	*	*	*	–	–	±100	±500	–		Enteritidis
91	Soil	*	*	–	*	*	*	*	–	–	±500	–100	–		Enteritidis
143	Soil	*	*	–	*	*	*	*	–	–	±500	±100	–		Enteritidis
206	Well	*	*	–	*	*	*	–	–	–	±100	+100	–	++(500)P	Enteritidis
213	Soil	*	*	–	*	*	*	–	–	–	–	–	–		Enteritidis
227	Well	*	*	–	*	*	*	–	–	–	+100	–	–		Enteritidis
294	Soil	*	*	–	*	*	*	–	*	–	+100	–	–	±(100)	Enteritidis
410	Well	*	*	–	*	*	*	*	–	–	±500	++100	–		Enteritidis
417	Well	*	*	–	*	*	*	*	–	–	+1000	–	–		Enteritidis
507	Soil	*	*	–	*	*	*	*	*	+	–	±500	++ 100	–	Paracoli
461	Soil	*	*	–	*	*	*	*	–	+	–	+100	–		Paracoli
424	Well	*	*	–	*	*	*	*	–	+	–	±500	–	+ colon ser. (500)	Paracoli
50	Well	*	*	–	*	*	*	*	*	+			–		Paracoli

Summary of Table I.

Total number of specimens	29
Number of wells	15
Number of soils	14

TYPES FOUND IN	DYSENTERY-LIKE BACILLI	PARA-ENTERITIDIS BACILLI	PARACOLON BACILLI	MORGAN BACILLI
Wells	3	9	2	2
Soil	9	4	2	0

+ = acid without gas.

* = acid with gas.

Note. The salicin positive, indol positive strains are considered as paracolon; the indol negative strains are classed as para-enteritidis. The bacilli fermenting acid without gas are for convenience thrown together into a "dysentery-like" group.

P = polyvalent antidysentery serum.

F = Specific Flexner antidysentery serum.

fered from the type organism in their behavior toward dulcite and salicin and in their agglutination reactions.

The paratyphoid-like organisms isolated both from wells and soil all belong to the para-enteritidis group. Their reactions, source, etc. are shown in the table. Thy differ from the *B. paratyphi A* in their power to ferment xylose; from the *B. paratyphi B* and *B. enteritidis* in their failure to attack dulcite (with one exception); and from the hog cholera bacillus in their ability to break down arabinose. They also fail to agglutinate in the higher dilutions with *B. paratyphi B*, *B. enteritidis*, or hog cholera sera. Only one strain (294) presents the characteristic reactions of the *B. enteritidis*, but even this strain is not agglutinated by the serum prepared from a typical strain in a dilution higher than 1: 100; although the titre of the serum was well over 2000. All the strains may therefore be considered members of the para-enteritidis group frequently present in excreta of domestic animals, particularly swine (Savage 1906-1910).

The dysentery-like organisms, or, to be more exact, the non-gas producing bacilli, were the forms most frequently isolated from soil and least frequently from wells. None of the strains was true to the dysentery type with respect both to fermentation and agglutination. Two strains were partially agglutinated in a 1: 1000 dilu-

tion of a polyvalent serum, and two others in 1: 500 of either polyvalent or specific Flexner serum. These agglutinated cultures all fermented sucrose and xylose and resembled, therefore, the Strong type. Three strains were agglutinated only in dilution of 1: 100 of the polyvalent or specific Flexner serum, although two of them were culturally identical with the Flexner bacillus. The other strains were not agglutinated at all. It is probable that these bacilli bear the same relation to the true dysentery organisms that the para-Gärtner bacilli do to the *B. enteritidis*. Organisms of this type have been found by Lewis (1911–1913), Alexander (1913), and others in stools of normal children and those suffering from diarrhea. As far as I am aware, they have not been reported from other sources; nor have they been shown to be definitely associated with any pathological conditions, although they are reported to be more frequently found in the excreta of children with diarrhea than in normal stools.

Paracolon bacilli are often found in both human and animal excreta. The usual strains ferment salicin and may or may not ferment dulcite. They also produce indol (Savage, 1906–1910) (Kligler, 1914). Their presence is of less significance than that of the other non-lactose fermenting bacilli, since they are as ubiquitous as the typical colon bacilli.

The Morgan bacilli are of interest because they have been definitely associated by the discoverer with the summer diarrheal diseases of children. Organisms of this type were isolated from two wells but not from the soil. Morgan and Ledingham (1909), Lewis (1911–1913), Alexander (1913), and others report the frequent presence of this bacillus in normal children and its more regular occurrence in children suffering from diarrhea. Morgan and Ledingham also found the bacillus in one cow out of 18 examined but not in the horse; while Lewis isolated it from 5 out of 20 mice. Whether it occurs in other animals is not known.

While it is hard to detect any absolute relations between the non-lactose fermenting bacilli and the sources of soil and water pollution, the fact does seem significant that the organisms most often found in wells are those more frequently encountered in the excreta of domestic animals, while the predominant sub-soil bacteria are more characteristic of human excreta. This relationship

between type and host is not definitely established, but is rendered highly probable by the results recorded in the literature. If the indication proves to be a fact, the results reported above suggest a surface origin of the well pollution, and a human origin of the subsoil contamination (the adjacent pits were indeed obviously responsible for the latter). A study of the non-lactose fermenting bacilli usually found in the intestines of domestic animals and human beings and their resistance to storage in soil and water might prove of decided value in tracing the source of pollution.

SUMMARY.

A study of the biological and serological characters of non-lactose fermenting bacilli isolated from polluted well waters and contaminated sub-soils is reported. It appears that the type of bacillus predominant in polluted well waters differed from that isolated from polluted sub-soils. The former belonged to the gas producing para-enteritidis group most common in the intestinal tract of swine, cattle, and other domestic animals. The latter, on the other hand, was a non-gas producing form resembling the bacillus found by Morgan, Lewis, and others in the stools of normal individuals, as well as in those of people suffering from diarrhea.

This relationship between the type of the non-lactose fermenting bacilli and host, though not definitely established, is suggestive. It is possible that a careful study of the non-lactose fermenting bacilli commonly found in the intestinal tracts of man and domestic animals would furnish the basis for an index of the source of pollution.

REFERENCES.

Adami and Chopin 1904 J. Med. Research, **11**: 469.

Alexander 1912–1913 Local Govt. Board, Med. Officer's Report, p. 381.

Kligler 1914 J. Infect. Dis., **15**: 187.

Lewis 1911–1913 Local Govt. Board, Med. Officer's Report, 1911–12, p. 265; 1912–13, p. 375.

Morgan and Ledingham 1909 Proc. Royal Soc. Med., **2**: Epidemiol. sect., 133.

Savage 1906–1910 Local Govt. Board, Med. Officer's Reports, 1906–07, p. 205, p. 253; 1907–08, p. 425; 1908–09, p. 316; 1909–10, p. 446.

Smith and Moore 1893 Additional investigation on swine diseases.

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THE INFLUENCE OF ETHER ANESTHESIA, OF HEMORRHAGE, AND OF PLETHORA FROM TRANSFUSION ON THE PRESSOR EFFECT OF MINUTE QUANTITIES OF EPINEPHRINE.

BY PEYTON ROUS, M.D., AND GEORGE W. WILSON, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Epinephrine is still employed with more or less danger and success to raise the blood pressure in states of collapse. The fact lends some practical interest to the observations to be described here. Their original purpose was to determine whether the response of the blood pressure to the intravenous injection of very small amounts of epinephrine might not be a useful indicator of diminished blood volume in cases suspected of hemorrhage. In the course of the experiments ether was noted to have a marked effect on the response. And this point will be the first taken up, since it has a direct bearing on the other work.

General Method.

Rabbits and dogs were used. Morphine and ether were the anesthetics given to the dogs, and paraldehyde, 1.5 cc. per kilo, was administered by stomach tube to the rabbits. A carotid artery was connected with a mercury manometer by the usual wash out cannula. The epinephrine injections were intravenous, into the external maxillary vein at its junction with the internal maxillary to form the external jugular. The flow in the jugular itself, from the internal maxillary, was utilized to flush the test doses into circulation, an important point in rabbits, since thus the introduction of a relatively considerable fluid bulk was avoided. The external maxillary ("posterior facial" in the rabbit) was dissected out for about 1.5 cm. above its junction with the internal maxillary ("anterior facial"), and a short, small cannula was introduced which carried on the end in the vessel a piece of narrow, soft rubber tubing about 0.8 cm. long. The end of the latter was pushed almost to the junction of the veins and held in place by a rubber-covered bulldog clamp, after which the sleeve of vessel wall was drawn up on the cannula and tied in place. When an injection was to be made, a hollow needle of exactly the length to reach to the end of the rubber tubing, and with a

rounded blunt tip, was thrust along the cannula, the bulldog clamp relaxed for an instant to permit its passage, and then closed on it again prior to the injection, which was practically instantaneous. In this way the test doses were delivered directly into circulation, and all injury to the vessel was prevented.

At the beginning of each experiment the minimum amount of epinephrine (Parke, Davis and Company) was determined which would bring about a well defined, if brief, rise of blood pressure, such as might conceivably be recognized in man by a coming through of the pulse beat in an arm compressed to just above the systolic pressure of the individual, as previously determined. It was found that in rabbits of 1,500 to 2,250 gm. our specimens of epinephrine caused regularly a pressure rise of 10 to 15 mm. of mercury when 0.5 cc. of a 1:1,000,000 dilution (the commercial solution diluted 1,000 times) was injected intravenously. This will be referred to hereafter as the minimum stimulative dose. Half the amount yielded only a negligible rise in pressure. The findings were identical in ten animals. The minimum stimulative dose caused no change in heart rate or amplitude, as indicated on rapid tracings, a fortunate circumstance, since thereby factors were ruled out which might have complicated the results.

No attempt was made to determine whether the minimum stimulative dose was as constant in dogs as in rabbits.

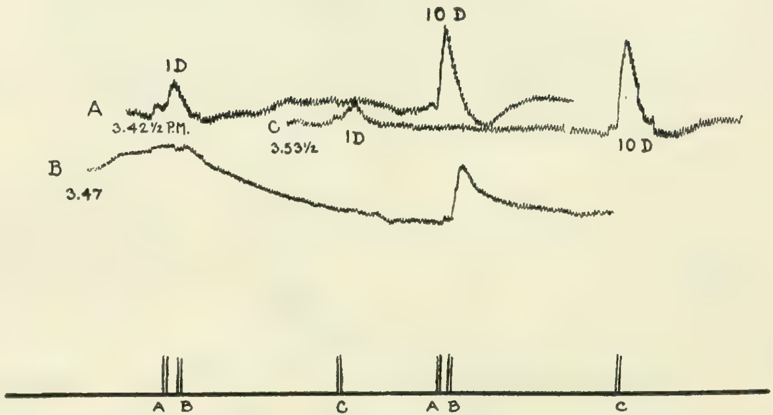
At the beginning of each set of observations, the normal pressure response of the animal to 1 minimum stimulative dose of epinephrine and to multiples of it was several times recorded on a kymograph. The injections were given in a fixed order and at fixed intervals, and this sequence was followed throughout the experiment, in order that a possible influence of one injection to affect the response to the next might be disposed of as a disturbing element. As a matter of fact, 10 minimum stimulative doses did not suffice to alter the response to a like amount given 2 minutes later. But with 100 minimum stimulative doses it was necessary to wait more than 5 minutes if an identical response was to be obtained from a second injection. Consequently such large doses were seldom employed.

Save in special instances the fluid bulk injected at each test was 0.5 cc. Epinephrine in a dilution of 1:1,000,000 in 0.9 per cent sodium chloride loses its pressor effect entirely in 3 to 4 hours at room temperature, and for this reason fresh dilutions had to be made in long experiments. Fortunately the 1:100,000 dilution deteriorates little, if at all, in this time. By means of tests with this and with new 1:1,000,000 solutions, a constant check was kept upon the results.

Effect of Ether.

In rabbits under the influence of paraldehyde, and in morphinized dogs, the amount of epinephrine required to cause a transient rise in the blood pressure of 10 to 15 mm. of mercury, that is to say 1 minimum stimulative dose, was found not to vary in successive tests on the

TEXT-FIG. 1. The effect of ether on the rise of blood pressure caused by epinephrine in a morphinized dog. 1 D, 10 D, 1 minimum stimulative dose, 10 minimum stimulative doses. The fluid bulk injected at each test was 1 cc. The base-line is the same for all the records. At *A*, when the blood pressure was normal, the animal was out of ether; at *B*, $11\frac{1}{2}$ minutes later, deep ether anesthesia had been produced; while at *C*, the animal was out of ether again. The dog weighed 4,750 gm. and had received 22 mg. of morphine sulfate subcutaneously about $4\frac{1}{2}$ hours prior to the first observation here recorded.



Text-Fig. 2. The effect of ether on the response to epinephrine in a rabbit under the influence of paraldehyde. *A*, prior to ether; *B*, deep ether anesthesia; *C*, out of ether. The animal weighed 2,250 gm. and was given 2.8 cc. of paraldehyde by stomach tube $2\frac{1}{2}$ hours before the first test here shown.

same animal within a period of 2 hours. When ether was given alone, or with the anesthetics already mentioned, the pressor response varied with the amount employed. Under light ether the response was similar to that with paraldehyde, but when the ether was pushed to the degree sufficient for operative procedures, 1 minimum stimulative dose of epinephrine aroused almost no response, and even that to 10 minimum stimulative doses was somewhat diminished (Text-fig. 1). When still more ether was given, so that the blood pressure fell 20 to 30 mm., the rise caused by 10 minimum stimulative doses was far less than the normal for the same animal, and, in rabbits at least, it was slow in appearing and prolonged—which doubtless accounts in part for the lessened amplitude (Text-fig. 2). The response to large doses was never affected to nearly the same degree as that to small ones.

In the deeply etherized animals there was often some cyanosis. But cyanosis alone, as brought about by a gradual stenosis of the trachea, did not affect the epinephrine response.

Effect of Hemorrhage.

There is much evidence to show that the blood pressure response to small doses of epinephrine is largely conditional on the state of contraction of the vascular muscle at the moment.[1] The cells are contracted more than usual, thus narrowing the stream bed, when the blood volume is diminished after a hemorrhage. These considerations led us to test the response in bled animals with a view to its possible clinical application. For there exists at present great need of some rapid indicator of lessened blood volume. According to Cannon and his coworkers,[2] and more especially to Govaerts,[3] an approximately normal blood count may be yielded by exsanguinated soldiers seen a few hours after their wound; and it may be impossible to distinguish clinically the collapse of lessened blood volume from that due to shock alone or an extending infection.[3]

[1] Cannon, W. B., and Lyman, H., *Am. J. Physiol.*, 1912–13, xxxi, 376.

[2] Cannon, W. B., Fraser, J., and Hooper, A. N., *J. Am. Med. Assn.*, 1918, lxx, 526.

[3] Govaerts, P., *Ambulance Ocean*, 1917, i, 355.

The extensive literature upon epinephrine contains almost no work concerning the effect of small amounts of the substance on a blood pressure lowered by hemorrhage. Hoskins, Rowley, and Rosser[4] bled dogs moderately (10 to 20 cc. per kilo of body weight) and injected an equal bulk of salt solution prior to tests with epinephrine. The blood pressure was not lowered by the bleeding and injection, and in view of the fact that no preliminary steps had been taken to deprive the animal's body of fluid reserves capable of making up the blood loss, it seems probable that only slight if any adjustment on the part of the vasomotor system could have been necessary. The authors conclude that "hemorrhage causes . . . . slight or no augmentation of the reaction to epinephrine."

In our experiments a reduction of the fluid reserves prior to the bleedings was deemed a primary consideration. Accordingly, both food and water were withheld from the animals during the 24 to 48 hours prior to operation. Relatively small bleedings in animals so treated brought about an enduring reduction of the blood pressure, whereas in those well watered this tended to return promptly to the normal after even considerable blood losses, and the effect of the epinephrine could not certainly be judged. The bleedings were accomplished through the free limb of the carotid cannula, which latter was disconnected from the manometer temporarily and flushed of anticoagulant. In reconnecting the manometer care was taken to lower its pressure beyond that which it was thought would now exist in the circulation. Thus any considerable entrance into the blood stream of the anticoagulant—half saturated sodium sulfate—was prevented.

A marked depletion was brought about by two to four successive bleedings. After each the response to epinephrine was several times tested. Then the blood bulk was restored, sometimes with horse serum or a 7 per cent solution of gum acacia in 0.9 per cent sodium chloride, but usually by returning the animal's own warmed blood, which had been kept from clotting by 0.5 per cent of sodium citrate. When the bleedings and reinjection were carried out rapidly, the blood pressure was usually restored to its original level. But when the animal had been kept in a severely depleted condition for 20 to 30 minutes, this result was not obtained, and even when fluid was injected in excess, the pressure remained low.

Paraldehyde and morphine were the anesthetics used, and in the manner already described. To control the results, ether was sometimes employed, always under careful restriction.

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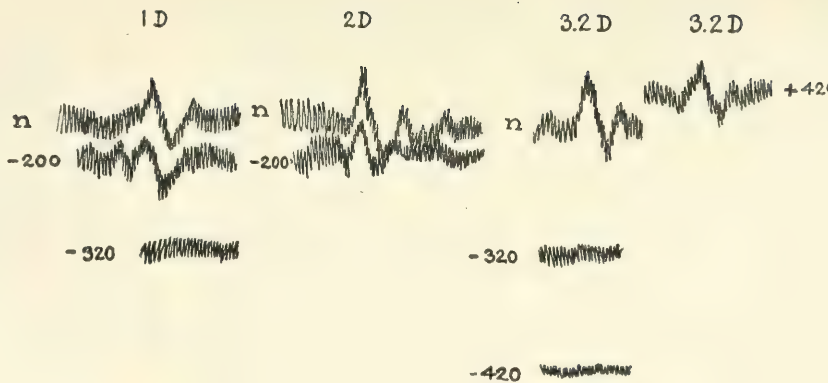
[4] Hoskins, R. G., Rowley, W. N., and Rosser, C., *Arch. Int. Med.*, 1915, xvi, 456.



Text-Fig. 3. The effect of hemorrhage and transfusion on the response to epinephrine of a morphinized dog. *n*, prior to bleeding; − 200, − 320, − 420, after the withdrawal of 200, 320, and 420 cc. of blood (in all); + 420 cc., after the reintroduction of the total blood removed. 1 D, 3.2 D, 1 minimum stimulative dose, 3.2 minimum stimulative doses respectively. The dog, weighing 11 kilos, received 66 mg. of morphine sulfate approximately 2½ hours before the first of the tests here shown.

The response to small doses of epinephrine was markedly affected by hemorrhages lowering blood pressure. Always a diminished pressure rise was noted, and the amount of the diminution was directly proportional to the lowering of the pressure consequent on the blood loss. When the pressure was very low, no response whatever was obtained from 1 or 2 minimum stimulative doses of epinephrine (Text-fig. 3), while sometimes 4 doses failed of effect. A response to 10 doses (0.5 cc. of a 1: 100,000 dilution) was never completely lacking, even in exsanguinated animals, but that seen was often sluggish and prolonged, as in animals heavily anesthetized with ether. That these results did not depend on mechanical inability of the cardiovascular system to raise the blood pressure, but to insufficient stimulus, was shown by the response to 100 minimum stimulative doses (0.5 cc. of a 1: 10,000 dilution), which was nearly always identical with the normal. Yet occasionally even this response was affected (Text-fig. 4).

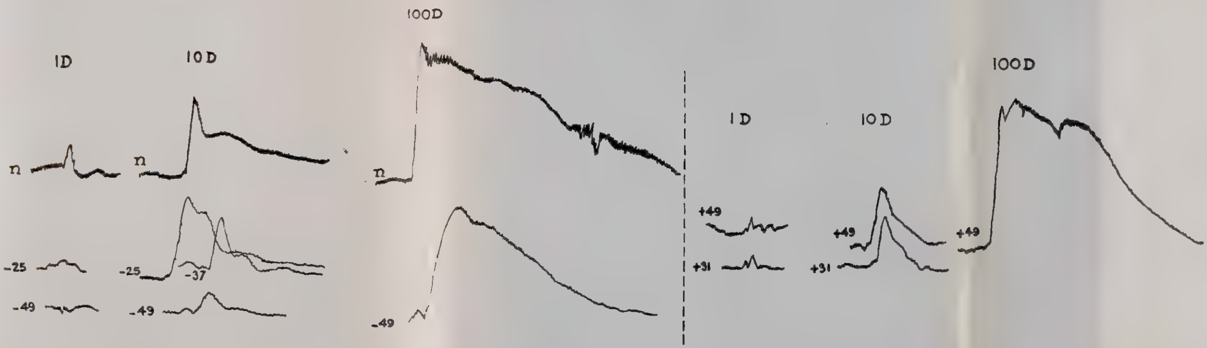
When the bleeding, testing, and reinjection of blood, or a substitute, were carried out briskly so that the blood pressure was low for only a few minutes, the response to epinephrine was not permanently impaired, but returned in proportion to the degree of restoration of the blood pressure. When the latter had reached the previous normal, the response was found to be approximately normal as well (Text-fig. 3). Prolonged depletion—for 20 minutes or more—had a lasting effect on the blood pressure which now could not be brought to the normal even by an excess of injected fluid. In such animals the response to epinephrine remained somewhat impaired (Text-fig. 4).

The results were the same in animals restored with horse serum or acacia solution instead of blood.

Effect of Plethora from Transfusion.

As a corollary to the findings under the condition of diminished blood bulk the influence of plethora was tested.

Rabbits were used and were given paraldehyde as in the previous experiments. A few observations were also made in animals given ether, but these were not satisfactory, owing to the great alterations in the state of anesthesia which were produced by the fluid injections. As a rule the fluid used, which was always

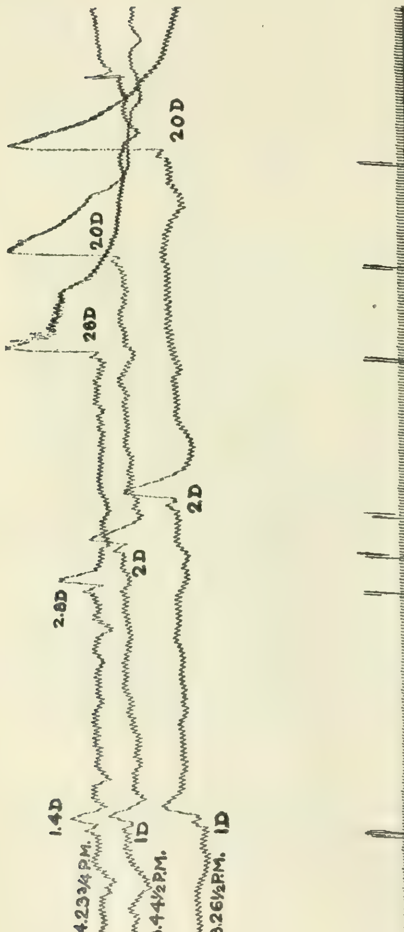


TEXT-FIG. 4. The effect of hemorrhage and transfusion on the response to epinephrine in a rabbit which had received paraldehyde. *n*, prior to bleeding; − 25, − 37, − 49, after the removal of 25, 37, and 49 cc. of blood (in all); + 31, + 49, after the reinjection of 31 and 49 cc. respectively of the citrated blood. The chart shows the damaging effect on blood pressure of 20 minutes exsanguination. The rabbit weighed 1,550 gm. and was given $1\frac{3}{4}$ cc. of paraldehyde approximately 3 hours before the first of the tests here recorded.

warmed to 38–40°C., consisted of the citrated whole blood of other compatible rabbits; but sometimes a 7 per cent acacia solution was employed. Each time that the bulk of circulating fluid was increased it was necessary, of course, to increase proportionally the test doses of epinephrine in order to obtain comparable concentrations in the blood stream. For the calculations that this entailed, the original content of blood was assumed to be 5.5 cc. for every 100 gm. of the animal's crude body weight.[5] With each increase in circulating fluid produced by transfusion, the test dose of epinephrine was increased correspondingly; and it was diminished again as the fluid was withdrawn by bleeding.

The increases in blood bulk did not affect the blood pressure of the animals that were anesthetized with ether, but in those under the influence of paraldehyde every considerable increment of fluid caused a pressure rise which was permanent during the short period of the experiment. For example, an initial increase in the blood volume of about one-eleventh was usually attended by a pressure rise of 10 to 15 mm. of mercury. The observations were made then upon animals which were not only plethoric but had an abnormally high blood pressure as well. The response to epinephrine under such circumstances was regularly a diminished one; and the degree of the diminution varied directly with the heightening of the blood pressure. A diminished response was obtained even when the test dose was far greater than that necessitated by the increase in blood bulk (Text-fig. 5). When the excess of fluid was withdrawn by bleeding, the response again assumed normal proportions. The findings in plethora can scarcely be due to the sodium citrate delivered with the transfused blood, for the same results were obtained with acacia solution, and exactly opposite ones were obtained when the animals were bled prior to transfusion. However, to settle the point, the influence of citrate by itself was tested. The amounts employed in the transfusions were found not to affect the epinephrine response.

[5] Boycott, A. E., and Douglas, C. G., *J. Path. and Bacteriol.*, 1909, xiii, 256. Frequent tests of the rabbit's blood volume with vital red by a method modified from that of Keith, Rowntree, and Geraghty (Keith, N. M., Rowntree, L. G., and Geraghty, J. T., *Arch. Int. Med.*, 1915, xvi, 547) have convinced us that this proportion closely approximates the real one.



Text-Fig. 5. The effect of high blood pressure with plethora from transfusion on the response to epinephrine in a rabbit. As in the preceding charts all the superimposed tracings are referable to the same base-line. The lowest tracing shows the results of tests under normal conditions; the next above the results of similar tests after the injection of 8 cc. of a 7 per cent solution of gum acacia in 0.9 per cent sodium chloride; and the highest the findings after a further 12 cc. of acacia solution. The acacia injections increased the blood volume by only $\frac{1}{11}$ and $\frac{3}{22}$ respectively of its original amount as calculated (5.5 per cent of the animal's crude body weight), yet they brought about notable rises in the blood pressure. For the tests in the normal condition and after the first acacia injection the same amounts of epinephrine were used; namely, 1, 2, and 20 minimum stimulative doses. After the second injection 1.4 times these amounts were employed, or nearly twice the increase that would have been proportional to the increase in blood bulk. The rabbit, weighing 1,600 gm., received 2 cc. of paraldehyde 3 hours prior to the first of the tests here shown.

DISCUSSION.

It does not lie within our intent to discuss at length the physiological basis of the facts here recorded. The problems presented are obviously complex. But the findings in etherized animals cannot be passed over without a reference to the study of Auer and Meltzer[6] on the response of skeletal muscle as affected by general anesthesia with ether. These authors find that the muscle contractility is much diminished by ether, as shown both by direct stimulation and when the nerve is stimulated. They further conclude that the drug has a curare-like action, inducing a resistance to the passage of stimuli through the nerve endings. It seems probable from our work that some at least of these effects are exerted on smooth muscle. For the rise in blood pressure caused by minute doses of epinephrine is the result of contraction of the vascular smooth muscle, through stimulation at the myoneural junctions (Cushny). And when the animal is under ether the rise is much less or may be absent. One is not warranted in assuming without further work that this is the result of a diminished contractility of the vascular muscle, brought about perhaps by a block at the myoneural junctions, yet certainly it is the most likely explanation. And the effect of the ether is as transient as in the case of skeletal muscle. In proportion as the animal comes out of the anesthetic, the response to epinephrine returns.

The influence of hemorrhage on the pressure response to epinephrine may perhaps be due in part to a slowed or otherwise altered circulation of the substance in the depleted animal, so that an effective concentration fails to reach at a single moment the walls of those portions of the stream bed which are chiefly engaged in maintaining blood pressure. Whether such a factor is really active we are not enabled to say. But it will scarcely suffice to explain the lack of response to three or four times the test dose that is effective in the unbled animal. And in this connection it may be pointed out that the test dose undergoes far less dilution when injected into the circulation of an animal after the blood bulk has been decreased by severe bleeding than when injected prior to the blood loss.

[6] Auer, J., and Meltzer, S. J., *J. Pharmacol. and Exp. Therap.*, 1913–14, v, 521.

The character of the response to epinephrine when the blood pressure is lowered but the blood bulk remains intact varies with the means used to bring about the condition. If it is consequent on peptone shock or anaphylaxis, a lessened pressor response is obtained[7] for reasons as yet unknown. If, on the other hand, it has been induced by pithing or by the administration of nitroglycerol, both of which cause a relaxation of the vascular muscle, an increased pressor effect, or the substitution of one for a depressor effect, may be the result.[1] Here apparently the response is directly related to the state of the vascular muscle. Whether the lessened pressor effect observed in animals with a blood bulk decreased by hemorrhage is dependent on the unusually contracted condition of the vascular muscle—as was our *a priori* assumption—cannot be decided from the present experiments. But certainly if this is the case one is forced to conclude that only when the contraction of the muscle is very marked does any noteworthy effect obtain. For moderate bleedings which do not cause a lowering of the blood pressure, though they undoubtedly must result in some vascular contraction for its maintenance, have slight or no influence on the response to epinephrine.

The findings in plethoric animals have an interest in this connection. The repeated small transfusions by which plethora was produced would supposedly tend at some period to cause a relaxation of the vessels in order to accommodate the increased blood bulk. With such relaxed vessels an increased response to epinephrine might be expected. But as a matter of fact, any changes observed were in the direction of a lessening of the response. The marked lessening noted when the plethora was great and the blood pressure high is a new illustration of the oft noted fact that epinephrine may have a relatively slight influence on a blood pressure already above the normal (Cushny).

Neither ether anesthesia nor hemorrhage appreciably affects the pressure response to large doses of epinephrine. Herein doubtless lies the explanation for the fact that the marked influence of these factors on the response to small amounts has been overlooked. Yet this influence, as now demonstrated, is not without practical impor-

[7] Simonds, J. P., *Arch. Int. Med.*, 1916, xviii, 848.

tance. If epinephrine is to be administered to hemorrhage cases or to patients in collapse under ether, it should be given in the knowledge that doses effective under normal conditions may now fail to elicit a vascular response. If administered in considerable amount and intravenously, a temporary response, differing little if at all from the normal, can be obtained. But small amounts given subcutaneously with a view to causing a pressure rise within physiological limits will almost surely fail of effect.

It is probable that many factors besides ether anesthesia and hemorrhage will be found to lessen the pressure response to small doses of epinephrine. The statement has already been made that anaphylactic shock and peptone poisoning will do so. And we have noted that the pressure response gradually though slowly diminishes in animals given paraldehyde and kept on the operating table, with a manometer connection, for from 6 to 8 hours. From all this a successful clinical use of epinephrine as an indicator of diminished blood volume would seem unlikely.

SUMMARY.

Ether anesthesia has a marked influence in diminishing the pressor response to minute amounts of epinephrine injected directly into the circulation. Hemorrhage also acts to lessen or abolish the response, and to a degree directly proportional to the lowering of the blood pressure it causes. In the exsanguinated animal an amount of epinephrine three or four times that sufficient to produce a pressure rise of 10 to 15 mm. of mercury under normal conditions, may be entirely without effect. The response to large doses, on the other hand, is uninfluenced by ether or hemorrhage.

The facts stated have a practical bearing not only on the employment of epinephrine to tide over collapse but on its possible utilization in the future to raise a low blood pressure to the normal height and maintain it during a considerable period. For the amount of epinephrine which under normal conditions will suffice to bring up the blood pressure may have little or no effect on an etherized individual or on one who has lost blood. The same difficulty will doubtless be encountered under other conditions.

In animals rendered plethoric by transfusion the response to small doses of epinephrine lessens in proportion as the blood pressure is increased by the plethora.

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ON THE OCCURRENCE OF DEGENERATIVE CHANGES IN THE LIVER IN ANIMALS INTOXICATED BY MERCURIC CHLORIDE AND BY URANIUM NITRATE.*

BY WILLIAM DEB. MACNIDER.

(*From the Laboratory of Pharmacology, University of North Carolina, Chapel Hill.*)

The following observations are based on the study of fifty-two intoxications by mercuric chloride and eighty-four intoxications by uranium nitrate. Dogs were employed for the experiments. In the animals intoxicated by mercuric chloride, the poison was administered by stomach tube in the dose of 15 mgs. per kilogram. In the uranium intoxications, the poison was given subcutaneously in doses varying from 4 to 6.4 mgs. per kilogram.

The experiments were terminated at different periods during the intoxication without employing an anesthetic. Such a termination has eliminated the acute degenerative changes in the liver which may develop very rapidly from the use of such an agent. The changes in the liver in both types of intoxications have shown great variation in their severity and the rapidity with which they occur.

Mercuric Chloride Intoxications.

All of the animals in this group, with eight exceptions, developed a severe gastroenteritis. The stools were frequent and contained blood and mucus. Twelve of the animals not only recovered from the gastroenteritis, but they failed to develop any delayed evidence of an intoxication. The remaining animals were either killed during the period of acute corrosive poisoning, or after having successfully passed through this stage, the experiments were terminated at different periods when the animals were suffering from the remote toxic effect of the poison.

* Aided by a grant from The Rockefeller Institute for Medical Research.

As a result of these studies, the following observations have been made:

1. There is no relationship between the severity of the gastroenteritis and the extent of the degenerative changes in the liver. The degenerative changes in the liver consist first, in a deposition of fat in the liver cells surrounding the central vein of the lobule. The severer changes which follow consist in cloudy swelling and necrosis of these cells, and an extension of the process to the periphery of the lobule. The invasion of the necrotic area by endothelial leucocytes is usually not a prominent reaction.

2. The more extensive liver degenerations have occurred in those animals that have recovered from the acute gastroenteritis but have later shown remote evidence of the intoxication by the development of an acute kidney injury.

3. A final group of animals has recovered from both the gastroenteritis and the kidney injury, but at a later period has shown the gradual or rapid development of an acid intoxication and a kidney injury of sufficient severity to induce an anuria. The pathology of the liver in this group of animals has shown two types of response. Evidence of repair has consisted in finding liver cells with mitotic figures and occasionally large cells with more than one nucleus. Connective tissue cells are more numerous than in normal liver tissue. In addition to these changes of a chronic character that indicate the repair of some previous injury, the liver has shown acute degenerative changes which are most marked in the midzone and periphery of the lobule. These changes have consisted in an acute necrosis which is preceded by fatty infiltration and edema. In the areas of necrosis, the sinusoids are large and distended with blood.

Uranium Nitrate Intoxications.

The earliest evidence of liver injury in uranium intoxications has consisted in the appearance in the liver cells of fat in the form of dust-like particles. This deposition is more marked in the cells immediately around the central vein than it is at the periphery of the lobule. Following this change, the cells show granular degeneration, an increase in size, and the deposition of fat in larger masses. The

later changes have consisted in marked cloudy swelling, followed by edema and necrosis. Such a termination is more marked near the center of the lobule than at the periphery. As the cytoplasmic degeneration progresses, fat appears in the cells in large droplets, and extends to the periphery of the lobule.

The rapidity of the development, and the severity of these changes have shown no definite dependence upon the size of the dose of uranium employed in the intoxication.

The severity of the degenerative changes in the liver and the amount of stainable fat present in the liver cells have shown a relationship with the age of the animal in which the intoxication is produced. The older animals have shown a susceptibility to uranium intoxication which has been expressed by the more rapid development of a liver degeneration and by these changes being more extensive than has been the case with the younger animals.

Associated with the occurrence of the degenerative changes in the liver, the animals develop an acid intoxication. Such an intoxication is of a severer type in old animals than in young animals.

At present an investigation is in progress which is concerned with the relationship of the liver injury induced by both mercuric chloride and uranium nitrate with the development of an acid intoxication.

[Reprinted from The Journal of Experimental Medicine, March 1, 1919, Vol. xxix, No. 3, pp. 283–304.]

EXPERIMENTS ON THE PRODUCTION OF SPECIFIC ANTI-SERA FOR INFECTIONS OF UNKNOWN CAUSE.

I. Type Experiments with Known Antigens—a Bacterial Hemotoxin (Megatheriolysin), the Pneumococcus, and Poliomyelitic Virus.

By PEYTON ROUS, M.D., OSWALD H. ROBERTSON, M.D., and JEAN OLIVER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 21, 1919.)

There are two generally recognized prerequisites to any attempt at the production in animals of an antiserum to combat an infectious disease. These are, needless to state, the isolation and the successful cultivation *in vitro* of the disease's causative agent. The difficulties here encountered in the case of some common and important maladies have absolutely held up all advances toward specific treatment.

The possibility of immunizing animals by direct injections of infected human tissues is one that must have occurred to many minds; and some attempts to employ the method were made prior to the general recognition that tissue itself acts to engender antibodies highly injurious to the species from which it is derived. With that recognition the method was given up, and it has not been revived. Yet there are noteworthy instances, such as the Pasteur treatment for rabies, which prove that tissue may contain an infectious agent in sufficient quantity to act as a practical antigen. And living tissues are superior in one respect to all other culture media, since organisms flourishing in them possess those pathogenic characters, loss of which in the test-tube sometimes limits, or indeed prevents, the development of antibodies in animals injected with cultures.

It has seemed to us possible that an antiserum produced by the injection of infected tissues might be rendered fit for employment in the species from which the tissues are derived by submitting it to a process of selective absorption, or exhaustion, with suspensions of

normal tissues. Such treated serum would, supposedly, be deprived of injurious tissue antibodies while retaining those directed against the infectious agent. A number of difficulties at once suggest themselves. It is well known that the absorption of antibodies is to a remarkable degree selective; and yet may not the repeated absorption of a polyvalent serum with tissue cells weaken notably the general content in antibodies? Will not toxic elements develop as a result of the incubation *in vitro* of mixtures of serum and tissue? And even should this not occur, is it really possible by the method of absorption to rid a serum of its anti-tissue potency so far as to render it harmless *in vivo*? May not the success of the absorptions be dependent on the employment of tissues derived from the same organ or organs used in the immunization? And finally, will not the method after all be more tedious than practical?

Some of these questions can be answered out of present knowledge. The recent work of many investigators on anaphylatoxins has shown that toxic elements are indeed engendered in incubated mixtures of tissue and its antibody, but only when complement is present.[1] In our selective absorptions inactivated serum could and should be used. The amount of tissue required to exhaust a serum of even high anti-tissue titer need not be great, since, as has been repeatedly shown in the typical case of red cells, many times the minimum hemolytic unit of amboceptor or agglutinin may be taken from a serum by a single unit of antigen. Furthermore, in the case of most sera, one can hope to use red corpuscles instead of tissues of the precise sort employed in the immunization. For much previous work has clearly demonstrated that only in certain special instances are specific cytotoxins the result of tissue injections.[2] Usually hemolysins, hemagglutinins, and serum precipitins alone develop.

In the work to be described our aim has been to determine the fundamental point, whether sera obtained by the immunization of animals with infected tissue of another species can, by the method of absorption, be rendered available for therapeutic use in the last mentioned species. No attempt has been made to devise practical

[1] See, for example, Friedberger, E., *Z. Immunitätsforsch.*, 1910, iv, 636.

[2] Pearce, R. M., *J. Med. Research*, 1904, xii, 1.

methods of absorption, to determine the least amount of tissue that will exhaust a serum, etc. For the purpose of type experiments sera have been selected containing different kinds of antibodies directed in all except one case—that of the chicken tumor—against pathogenic agents already isolated and well studied.

Selective Absorption Applied to an Antitoxic Serum.

The simplest case, that of an antitoxic serum, was first taken up. In view of the possible importance of "anaphylatoxins" engendered in the incubated mixture of serum and tissue as above mentioned, it seemed best to work with the animals most susceptible to such poisons; namely, guinea pigs. Furthermore, it was desirable to employ, if possible, a toxin of which the neutralization with antitoxin could be observed *in vitro* as well as *in vivo*. Both conditions are met by the hemolysin produced by *Bacillus megatherium* and first studied by Todd.[3] Todd demonstrated that the lysin is a true toxin, against which an antitoxin can be readily produced in guinea pigs, rabbits, and goats. His work has been fully confirmed.[4] *Bacillus megatherium* produces its toxin only when grown *in vitro* under special conditions, and although the toxin will kill guinea pigs, the organism itself is practically non-pathogenic. For this reason infected tissues could not be obtained for the immunizations required by our plan. Normal tissues mixed with the toxin might perhaps have been used, but the latter by itself, when injected locally, causes a violent inflammatory reaction. It was decided, on this account, to inject the toxin and the tissue used for immunization at separate sites and at different times.

Characters of the Megatheriolysin.—Three strains of *B. megatherium* were obtained from the American Museum of Natural History through the kindness of Professor Winslow. Only one produced any noteworthy hemolysin when cultivated in the special bouillon recommended by Todd.[3] This produced a lysin of such strength that 1 cc. of the Berkefeld filtrate of a 7 day culture, when in-

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[3] Todd, C., *Lancet*, 1901, ii, 1663; *Tr. Path. Soc. London*, 1902, liii, 196.

[4] Dreyer, G., and Blake, J., *Lancet*, 1904, ii, 408. Craw, J. A., *Proc. Roy. Soc. London, Series B*, 1905, lxxvi, 179. Vincent, H., *Compt. rend. Soc. biol.*, 1909, lxvii, 195.

jected intravenously into a 400 gm. guinea pig, regularly caused intense hemolysis and hemoglobinuria, with death in a few hours at most. Intraperitoneally 2 cc. produced death quite as rapidly from diffuse petechial hemorrhages, first into the mucosa of the small intestine, and thence into the lumen of the intestine, which became distended with fluid blood. The uterus, large intestine, and stomach sometimes showed petechiæ, usually scattering. Despite the striking local lesions there was no evidence of intravascular hemolysis and never any hemoglobinuria, even when death occurred slowly. When given subcutaneously the lysin caused a wide area of necrosis.

The strain of *B. megatherium* furnishing the lysin was cultivated in quantity in Todd's medium, and at the end of 7 days the culture fluid was centrifuged and passed through a Berkefeld filter, after which the filtrate was tested for sterility, tubed, the tubes sealed with paraffin, and stored in the dark at 2–3°C. Under these conditions the lysin was found to retain practically all its activity for months, a great advantage, since portions of the same filtrate could be used both for the immunization of animals and for tests of the antisera that they yielded.

Immunization of Animals.

The lysin is most injurious to guinea pigs, yet large amounts are ill tolerated by rabbits or goats. The normal serum of both these animals has some slight neutralizing activity for the lysin, as shown by the ability to prevent hemolysis of guinea pig corpuscles. Attempts were made at first to immunize rabbits. A number of these animals were given six intraperitoneal injections at 6 day intervals of defibrinated guinea pig blood plus suspensions of ground liver and spleen, followed 2 days later by intraperitoneal injections of 1 cc. of the megatheriolysin. The animals were bled 7 days after the last lysin injection. They stood the immunization badly; all lost weight, and several died. Nevertheless, as will be seen, the survivors elaborated a well marked antitoxin.

A goat was immunized by separate subcutaneous injections of tissue and lysin according to the method just outlined. The finely ground liver, spleen, and kidney of guinea pigs, and the defibrinated blood were mixed and used. The amount of megatheriolysin given was gradually increased from 0.5 to 10 cc., diluted always with salt solution. This caused boggy swellings which were slow to subside. 7 days after the last of the six lysin injections the goat was bled, and the serum was tubed without the addition of preservatives and left in the ice box. The rabbit serum was similarly treated.

Method of Exhaustion with Red Cells.

The serum was inactivated at 56°C. for ½ hour just prior to use. Guinea pig red cells taken into citrate were thrice washed in ½ per cent gelatin Locke's solution under aseptic conditions. In ordinary salt solutions guinea pig corpuscles

are prone to break down when washed; but the addition of a little gelatin to the washing fluid will prevent this.[5] After the last washing the cells were packed in graduated tubes, their bulk was noted, all possible fluid pipetted off, and the serum to be exhausted poured on, the tube corked, inverted, and gently shaken to suspend the cells. The suspension was now warmed to 38°C. in the water bath, incubated, centrifuged at high speed, and the serum transferred to a fresh lot of cells. The period of incubation varied with the degree of agglutination of the cells. When they fell out promptly into a solid mass such as could not be broken up without hemolysis, incubation for more than a few minutes was manifestly useless. But often after one or two absorptions the cells tended to remain in suspension, and the incubation period was lengthened to an hour. Finally, when the suspended cells showed no trace of agglutination, incubation was continued for as long as 2 hours in some instances.

No preservative was added to the mixture of serum and cells, but great care was taken to assure its sterility. The special corks for centrifuge tubes, elsewhere described,[5] were an aid in this connection. After the last absorption cultures were regularly taken. These showed an entire absence of infection, which may perhaps be attributed as much to the frequent centrifugation at high speed to which the serum was subjected as to our technique.

With repeated absorption there was a slight unavoidable loss of serum, and a slight dilution of it also occurred, owing to the remnant of gelatin Locke's solution introduced with the cells. In testing the relative potency of the unexhausted and exhausted serum usually no correction was made for this dilution of the latter.

Specimen Experiments—Rabbit Serum.

Experiment 1.

For this and all the subsequent tests the sera were inactivated at 56°C. for ½ hour. Whenever the period of incubation of the *in vitro* mixture is not specifically mentioned it was for 2 hours at 38°C. Readings were made after the tubes had stood over night in the ice box.

Hemolytic Activity of the Megatheriolysin.—This was determined as follows: 0.25 cc. of megatheriolysin in graded dilutions + 0.25 cc. of 0.9 per cent sodium chloride + 0.25 cc. of a 5 per cent suspension of washed guinea pig red cells.

Lysin strength*..........	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$
Hemolysis..............	C.	C.	C.	C.	+++	Ft. Tr.	0

* The lysin strength is expressed in terms of the undiluted material.

Antilytic Titer of Normal Serum.—The antilytic titer was tested of the inactivated sera of four normal rabbits, as contrasted with that of an animal repeatedly injected with megatheriolysin and guinea pig tissues. Mixtures were made of

[5] Rous, P., and Turner, J. R., *J. Exp. Med.*, 1916, xxiii, 219.

the sera in graded dilutions with a fixed amount of megatheriolysin, guinea pig red cells were added, and incubation was done. The amount of lysin in each tube was more than eight times that necessary under ordinary conditions for complete hemolysis of the corpuscles (*vide supra*). Subsidiary tests which need not here be given in detail showed that rabbit serum exercised its whole neutralizing effect on the lysin practically at once when mixed with it at room temperature. Consequently no interval was allowed to elapse before the red corpuscles were added.

0.25 cc. of rabbit serum in graded dilutions + 0.25 cc. of $\frac{1}{5}$ strength megatheriolysin + 0.25 cc. of 5 per cent guinea pig red cells.

Serum.		Serum dilution.							
		0	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Normal.	A	++	+++	++++	Alm. C.	C.(?)	C.	C.	C.
	B	+	+	++	+++	++++	Alm. C.	"	"
	C	+	+	++	++	+++	" "	"	"
	D	+	++	++	++++	++++	" "	"	"
Immune		0	0	0	Tr.	+++	" "	"	"

It is evident from this experiment that the normal rabbit sera possessed some power to prevent destruction of corpuscles by the megatheriolysin. But the immune serum conferred at least eight times as much protection, as shown by comparing its effect, when diluted, with that of the concentrated normal sera. The failure of the immune serum to protect to the same proportional degree in the higher dilutions is attributable to the presence in megatheriolysin of several lytic components[3] against all of which doubtless the antiserum had not the same relative activity.

Anti-Guinea Pig Titer of the Immune Serum.—This was well marked as a result of the repeated injection of the immunized rabbit with guinea pig tissue. A precipitin was present effective against dilutions of guinea pig serum up to and including 1:256 when an equal bulk of the concentrated immune serum was mixed with it. Tests for hemolysis and agglutination were made as follows:

0.25 cc. of serum dilution + 0.25 cc. of 1 in 10 guinea pig complement + 0.25 cc. of 5 per cent guinea pig red cells.

	Serum dilution.											Complement + red cells + 0.25 cc. of salt solution.
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1,024	
Hemolysis	0	+	+++	++	±	±	Tr.	Tr.	Ft. Tr.	0	0	0
Agglutination	C.	C.	C.	C.	C.	C.	++++	±	Tr.	0	0	0

The low hemolytic titer was doubtless due in part to serum precipitation which took place in the mixtures, for complement is absorbed during such precipitation, as is well known. But it is also explained by the close biological relationship of rabbit and guinea pig which renders difficult the production of antibodies in the one against the other. The fact should also be recalled that specific hemolysins as a rule act but weakly when complemented with serum from the species furnishing the test cells. Yet it was deemed best to use such complement in our tests, since it would be the only one present during *in vivo* experiments.

Selective Absorption of the Anti-Guinea Pig Elements.—3 cc. each of the four normal sera and the immune serum above mentioned, all inactivated, were mixed respectively with 1.5 cc. of sedimented guinea pig red cells, incubated for 1 hour, and centrifuged, and the serum was transferred to a fresh portion of red cells. In the case of Normal Sera C and D only 0.5 and 0.65 cc. respectively of red cells were employed in the second absorption, while for the others 1.5 cc. were used as before. Incubation again was for 1 hour. In the first mixture of immune serum and cells a moderate agglutination was to be seen. None of the other mixtures ever showed the least trace of clumping.

Tests were made after the second absorption to determine how completely hemolysin and agglutinin had been removed from the immune serum. No trace of either was found. In view of these results tests to show whether the normal rabbit sera had been completely exhausted were deemed unnecessary, since such sera when untreated are almost devoid of antibodies for guinea pig red cells.

Antilytic Titer of the Treated Immune Serum.—0.25 cc. of immune serum in graded dilutions + 0.25 cc. of ½ megatheriolysin + 0.25 cc. of 5 per cent guinea pig red cells.

Serum.	Serum dilution.							
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Untreated	0	0	0	Tr.	+++	Alm. C.	C.	C.
Exhausted	0	0	0	+++	Alm. C.	C.	"	"

The exhaustion with red cells had but little diminished the antimegatheriolytic titer of the serum.

In Vivo Tests of the Neutralization of Megatheriolysin with Exhausted Serum.—A number of mixtures containing 1 cc. of undiluted megatheriolysin with 0.75 cc. of serum or salt solution were incubated 2 hours, and 1.5 cc. of each were injected directly into the ear vein of a guinea pig. The method of injection has been described elsewhere.[6] The long period of incubation was unnecessary, since neutralization of the lysin is, as has been stated, practically instantaneous at room temperature.

[6] Rous, P., *J. Exp. Med.*, 1918, xxvii, 459.

Guinea Pig No.	Weight.	Mixture used for injection.	Result.
	gm.		
1	350	Exhausted immune serum + lysin.	Remained well.
2	350	Untreated " " + "	Died immediately after injection.
3	375	Exhausted normal serum (No. 1) + lysin.	Died 6 hrs. later; intense hemolysis; hemoglobinuria.
4	350	Exhausted normal serum (No. 2) + lysin.	Died 10 hrs. later; intense hemolysis; hemoglobinuria.
5	375	Exhausted normal serum (No. 3) + lysin.*	Died 40 hrs. later; intense hemolysis; hemoglobinuria.
6	375	Exhausted normal serum (No. 4) + lysin.	Died 10 hrs. later; intense hemolysis; hemoglobinuria.
7	350	Salt solution + lysin.	Died 13 min. later; intense hemolysis; at autopsy characteristic lesions.
8	350	" " + "	Died 12 hrs. later; intense hemolysis; hemoglobinuria.

* Part of injection material lost.

All the animals that succumbed, except No. 2, showed the lesions already described as characteristic of the megatheriolysin. In this case death was practically immediate, and there were no gross lesions except an almost complete intravascular hemagglutination, caused, of course, by the untreated immune serum, and without doubt the cause of death.

Experiment 2.

Another less comprehensive experiment with immune rabbit serum will be briefly quoted, since in addition to showing the antitoxic power of the exhausted serum it affords an interesting contrast between the lesions caused by the megatheriolysin, as such, and mixtures of the lysin with unexhausted serum.

The serum of an immunized rabbit was incubated as already described with two successive batches of washed guinea pig red cells. *In vitro* tests showed that the hemolysin and agglutinin were thus removed, whereas the antilysin was practically as strong as ever. The following mixtures were now made: (*a*) 2 cc. of megatheriolysin + 1.25 cc. of serum or salt solution; (*b*) 4 cc. of megatheriolysin + 2.35 cc. of serum or salt solution. After 2 hours incubation at 38°C. the whole of each mixture was injected into the peritoneal cavity of a guinea pig.

Guinea Pig No.	Weight.	Mixture (a). Lysin +	Result.
	gm.		
9	480	Exhausted immune serum.	Remained well; no anemia.
10	480	Untreated " "	Died after 48 hrs.; extreme anemia; hemoglobinuria.
11	480	Salt solution.	Died after 4½ hrs.; lesions characteristic of the megatheriolysin.
		Mixture (b). Lysin +	
12	500	Exhausted immune serum.	Remained well; no anemia.
13	500	Untreated " "	Died after 48 hrs.; progressive anemia; hemoglobinuria.
14	520	Salt solution.	Died after 1½ hrs.; lesions characteristic of the megatheriolysin.

The hemoglobin percentage in the blood of the surviving animals was followed for some days.

At autopsy the animals receiving megatheriolysin + salt solution presented the findings already described as characteristic after intraperitoneal injections. The small intestines were distended with blood from many fine hemorrhages into the mucosa. Other hemorrhages were present in the walls of the large intestine and stomach. The blood remaining in the vessels was unclotted, greatly concentrated, but unhemolyzed and unagglutinated. There was never any hemoglobinuria. The lesions from mixtures of the lysin with unexhausted serum were entirely different, being those characteristic of a serum hemolysin. A severe progressive anemia developed, accompanied by hemoglobinuria, and the blood specimens showed marked agglutination and many shadows. At autopsy there was no trace of intestinal hemorrhages such as result from the megatheriolysin. The spleen was greatly enlarged and crowded with phagocytes filled with red cells. There were also scattered ecchymoses on the pleuræ, diaphragm, and parietal peritoneum—a lesion never observed as the result of the megatheriolysin but commonly produced by a serum hemolysin. The conclusion is unavoidable that the animals had been saved from the action of the megatheriolysin only to succumb to that of the serum hemolysin.

Specimen Experiment—Goat Serum.

Experiment 3.

A freshly prepared lysin was employed for the work. Its titer diminished so little in the course of the 6 weeks during which a goat was repeatedly injected with it that tests made of the neutralizing activity of the serum of the animal before and after the immunization can be directly compared.

Hemolytic Activity of the Lysin.—0.2 cc. of megatheriolysin in graded dilutions + 0.2 cc. of 5 per cent guinea pig red cells.

Hemolysis.	Lysin strength.							
	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640
At first test	C.	C.	C.	C.	C.	Alm. C.	+++	Tr.
6 wks. later	"	"	"	"	Alm. C.	" "	+	"

Antilytic Titer of the Goat Serum before and after Immunization.—These tests were made at the same time as those of the megatheriolysin just quoted. 0.25 cc. of goat serum in graded dilution + 0.25 cc. of ⅛ strength megatheriolysin + 0.25 cc. of 5 per cent guinea pig red blood cells.

Serum.	Serum dilution.									Whole strength serum + red cells + salt solution.
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	
Prior to immunization	+++	+++	++++	Alm. C.	Alm. C.	Alm. C.	C.	C.	C.	0
After immunization (6 wks. later)	0	0	0	±	C.	C.	"	"	"	
Serum of a normal goat (control)	Alm. C.	C. (?)	C.	C.	"	"	"	"	"	

The serum of the normal control was obtained and tested at the same time as the immune serum.

Plainly the immunization with megatheriolysin had increased the antilytic titer of the goat serum.

Anti-Guinea Pig Elements and Their Selective Absorption.—Preliminary tests showed that the serum of the immunized goat contained powerful antibodies for guinea pig red cells, as would naturally follow from the repeated injection of the animal with tissues of this species. An attempt was made to absorb the antibodies from a portion of the serum, and the titer of the exhausted specimen was then compared with that of an untreated portion. The normal control serum was not submitted to absorption because it was found to be harmless to guinea pigs when given in the doses required by our experiments.

For the purposes of exhaustion 17 cc. of the immune serum were incubated for 1 hour with 5 cc. of washed guinea pig red cells, the mixture was centrifuged, and

the serum transferred to more red cells and incubated again. This was done five successive times. In the first mixtures the red cells were only moderately agglutinated, but in the later ones clumping became much more marked owing doubtless to the absorption of proagglutinoids, which at first had hindered agglutination.

Mixture.	Hemagglutination.
17 cc. of serum + 5 cc. of red cells incubated 1 hr. and	Moderate.
serum transferred to 4.25 cc. of red cells, incubated 1 hr. and	"
" " " 7.6 " " " " " 1 " "	Strong.
" " " 7.3 " " " " " 1 " "	Almost massive.
" " " 5.2 " " " " " 1 "	Moderate.

Only 13.5 cc. of serum were finally recovered. The diminution in volume was due to a retention of the fluid amidst the agglutinated red cells. The latter, when clumped by the serum, occupied a greater space than when simply sedimented in salt solution.

A comparison was now made of the hemolytic and agglutinative titer of the exhausted and unexhausted immune serum, and of the normal control serum. 0.2 cc. of inactivated goat serum in graded dilutions + 0.2 cc. of 1 in 10 guinea pig complement + 0.2 cc. of 5 per cent guinea pig red cells were used.

	Serum dilution.												
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1,024	1/2,048	1/4,096
Serum.	Hemolysis.												
Untreated normal..........	+−	Tr.	Tr.	Ft. Tr.	0 (?)	0	0	0	0	0			
Untreated immune........	Ft. Tr.	++−	++	+	Tr.	Ft. Tr.	Ft. Tr.	−	0	0			
Exhausted immune........	No hemolysis.												
Serum.	Agglutination.												
Untreated normal..........	No agglutination.												
Untreated immune........	+++	+	++	C.	C.	C.	C.	C.	C.	++	+	Tr.	0
Exhausted immune........	+++	+++	+++	++	+	±	0	0	0	0			

Agglutination was read in the same mixtures as hemolysis. With rabbit complement the untreated immune serum was found to be far more hemolytic than with guinea pig complement as here shown. Precipitation was observed in the hemolytic mixtures containing immune serum in dilutions up to 1: 64, and to this is attributable the Neisser-Wechsberg phenomenon observed in the hemolytic tests of the untreated specimen.

Antilytic Titer of the Treated Immune Serum.—0.25 cc. of serum in graded dilutions + 0.25 cc. of ⅛ megatheriolysin + 0.25 cc. of 5 per cent guinea pig red cells.

Serum.	Serum dilution.								
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Untreated immune	0	0	0	±	C.	C.	C.	C.	C.
Exhausted "	0	0	0	++	"	"	"	"	"
Untreated normal	Alm. C.	C. (?)	C.	C.	"	"	"	"	"

The repeated absorption of the immune serum with large amounts of red cells (29.35 cc. of the latter in all, as against only 17 cc. of serum) was found scarcely to affect the antimegatheriolytic titer, which remained more than eight times that of the normal serum.

In Vivo Tests of the Neutralization.

(*a*) *Intraperitoneal Injections.*—Mixtures of 2 cc. of megatheriolysin (or salt solution) with 2.5 cc. of serum (or salt solution) were incubated 1 hour at 38°C. and injected into a number of guinea pigs of 400 gm. weight.

Guinea Pig No.	Mixture injected.	Result.
15	Normal serum + salt solution.	Remained well; no anemia.
16	0.9 per cent salt solution + lysin.	Died after 1½ hrs. with characteristic lesions.
17	Normal serum + lysin.	" " 12 " " " "
18	Unabsorbed immune serum + lysin.	" " 53 " " progressive anemia and hemoglobinuria.
19	Absorbed immune serum + lysin.	Remained perfectly well save for a slight anemia soon repaired.

The order of injection was as follows: Nos. 15, 17, 19, 18, 16.

The findings at autopsy were similar to those of Experiment 2 with rabbit serum, as already described. All the animals that died, save No. 18, showed

lesions characteristic of the action of the megatheriolysin. In the guinea pig mentioned the lesions were of a different sort, being such as are caused by a specific hemolysin; and no trace of injury from the megatheriolysin was to be seen.

(*b*) *Intravenous Injections*.—Mixtures of 1 cc. of lysin (or salt solution) with 0.8 cc. of serum (or salt solution) were incubated 1 hour at 38°C., and 1.5 cc. of each mixture were injected into the ear vein of a guinea pig.

Guinea Pig No.	Mixture injected.	Result.
20	Normal serum + salt solution.	Remained well; no anemia.
21	" " + lysin.	Died after 42 hrs. with progressive anemia and hemoglobinuria.
22	Salt solution + "	Died after 47 hrs. with progressive anemia and hemoglobinuria.
23	Untreated immune serum + lysin.	Died after 8 hrs.; prostrated at once; hemoglobinuria.
24	Exhausted immune serum + lysin.	Remained well save for a moderate anemia.

Guinea Pig 20 weighed 425 gm., the others 450 gm.

The order of injection was as follows: Nos. 24, 23, 21, 20, 22.

Comment.

It is clear that by the method of selective absorption an antitoxic serum strong in tissue antibodies can be deprived of the latter to such extent as to be converted from a highly injurious agent into one capable of saving life. Indeed such a serum submitted to absorption five successive times, with a total bulk of corpuscles almost twice its own volume, was found to retain practically all its titer in antitoxin (Experiment 3).

A few of the guinea pigs receiving mixtures of exhausted serum and megatheriolysin were temporarily prostrated, lying on the side, and twitching, but they soon got to their feet and showed no permanent injury. The prostration occurred as frequently after the injection of normal serum as of that from animals immunized against guinea pig tissues. It is not possible to say whether the symptoms were due to "anaphylatoxin," or resulted from struggle under durance in connection with the rapid intravenous injection of a relatively large amount of foreign fluid. No such symptoms were ever observed after intraperitoneal inoculations.

The animals saved by the action of exhausted immune serum gave no evidence of injury to liver, spleen, or kidney, such as might perhaps have been expected in view of the fact that suspensions of these organs mixed with defibrinated blood were employed in the immunization. However, the only urine test made was for hemoglobin. Some of the guinea pigs receiving exhausted serum developed a slight or moderate anemia which was slow to appear and was most marked 4 or 5 days after the injection. Special experiments which need not here be cited in detail showed convincingly that the blood injury was due, not to unneutralized megatheriolysin, but solely to insufficient exhaustion of the serum with red cells, as proved by the persistence in it of hemagglutinins. When the absorptions were continued until all hemagglutinins had been removed, the serum was harmless to the blood. For example, it was found that the serum of Experiment 3 after seven absorptions no longer possessed hemagglutinins or produced anemia, whereas after only five absorptions it had both these characters as our protocol shows. The point is an important one, suggesting that the complete absence of hemagglutinins may be taken as the index to when exhaustion of a serum is complete. This indicator has been adopted, in much of our later work, and properly, as the results show. Hemagglutinins are far stronger than hemolysins in most sera resulting from prolonged immunization with animal tissues, and persist long after the latter have been removed by absorption.[7]

Selective Absorption Applied to an Antipneumococcus Serum.

The results of the work with an antitoxic serum were so encouraging that experiments were begun with sera of other types. It was highly desirable that they should be developed through the actual employment of infected tissues as antigen. The pneumococcus was selected for some of the tests, and attempts were made to immunize dogs against the organism by means of injections with the tissues of rabbits dying of pneumococcus septicemia. The difficulties encoun-

[7] An exception is to be noted in the case of anti-chicken sera from the goose and rabbit. These regularly contain hemolysins in fair quantity but only weak hemagglutinins.

tered illustrate strikingly the differences which may exist in the pathogenicity of a microorganism growing *in vivo* and *in vitro*.

Dogs possess a considerable resistance to the pneumococcus compared with some other species, as is well known; and they will often withstand the intravenous injection of several cubic centimeters of a bouillon culture fatal in minute quantity to mice. Intraperitoneal inoculations are even better borne. Nevertheless the immunization of dogs against the pneumococcus by intraperitoneal injections of blood or other tissues from rabbits moribund with pneumococcus septicemia proved well-nigh impossible, because of the high virulence of the antigen and our inability to standardize that derived from different rabbits. Small amounts of the infected tissue caused the dogs to die with a pneumococcus septicemia. Not infrequently they withstood a number of injections, only to succumb to one which was, quantitatively speaking, inconsiderable. In order to avoid this the infected rabbit tissue was heated *in vitro* at temperatures between 40° and 50°C. prior to injection, and the number of living organisms was thus reduced, as cultures showed, from millions to but a few per cubic centimeter. Still the injections often resulted fatally. Separate intraperitoneal inoculations of pneumococcus cultures and of normal tissues gave better results, but pneumococcus peritonitis so often ensued that at length separate subcutaneous inoculations were decided upon. These were carried out over a period of several months and a serum was finally obtained of sufficient antipneumococcus and anti-tissue titer to be suitable for experiments on selective absorption. We can confirm the observation of Nuttall[8] and Doerr and Moldovan[9] that antibodies of high titer are with difficulty elicited in the dog, as a response to immunization.

The plan of the experiments required yet a further alteration. Rabbits were found to vary so markedly in their resistance to the pneumococcus that very many would have been required had they been used as test animals in protection experiments with the antipneumococcus serum. It was decided, on this account, to exhaust the dog serum with rabbit red cells, in the manner that had proved successful with antimegatheriolytic serum and guinea pig cells, but to

[8] Nuttall, G. H. F., Blood immunity and blood relationship, Cambridge, 1904.

[9] Doerr, R., and Moldovan, J., *Z. Immunitätsforsch., Orig.*, 1910, vii, 223.

carry out protection tests with mice instead of rabbits. Mice were found to tolerate well the intraperitoneal injection of normal dog serum in the amount necessary for the work.

Immunization of Animals.—Nine dogs weighing from $8\frac{1}{4}$ to 13 kilos were injected intravenously with amounts varying from 0.5 to 1.25 cc. of the mixed citrated blood of three rabbits moribund with pneumococcus septicemia. The organism was of Type I (Neufeld strain), and the infected blood was preserved in the frozen condition for 4 days prior to use. Five of the dogs died of pneumococcus septicemia within a few days after the injection. The surviving four animals received injections of antigen at intervals of 7 days for more than 2 months. At first citrated septicemic blood was given intraperitoneally. This was badly tolerated, so recourse was had to inoculations with normal tissue and bouillon cultures of the pneumococcus, given at separate subcutaneous sites and on different days. The normal tissue consisted of suspensions of finely ground rabbit liver, spleen, and kidney mixed with defibrinated blood. Kidney tissue in special was employed, with the object of obtaining a serum that would be nephrotoxic. None of the dog sera acquired a demonstrable nephrotoxin, however, though hemolysins and hemagglutinins soon developed, and also weak agglutinins for the pneumococcus. The two most highly immunized dogs (Dogs A and B), as judged by these features, were bled for serum 9 days after the last pneumococcus injection.

Exhaustion of the Serum.—The method of selective absorption was that already described in connection with antimegatheriolytic serum. Rabbit red cells, thrice washed, were packed by rapid centrifugation, all possible fluid was removed, and the cells were mixed and incubated with the serum under test. The latter was in this way exhausted by contact with several successive portions of cells.

Experiment.—The sera of two immunized dogs and three normal controls were inactivated at 56°C. for $\frac{1}{2}$ hour, and portions of all were submitted to an exactly similar exhaustion with red corpuscles. The incubation period was 1 hour with each successive batch of cells.

Mixture.	Hemagglutination.		
	Immune sera.		Normal sera.
	A	B	
26 cc. of serum + $3\frac{1}{3}$ cc. of red cells, incubated 1 hr. and	Massive.	Heavy.	
serum transferred to $3\frac{1}{3}$ cc. of red cells; incubated 1 hr.	Moderate.	Moderate.	
" " " 4 " " " " " 1 "	Slight.	Slight.	0
" " " 4 " " " " " 1 "	Tr.	Ft. Tr.	

Cultures taken after the last absorption showed all the sera to be sterile.

Anti-Rabbit Titer of the Immune Sera. Hemolysis.—0.2 cc. of inactivated serum in graded dilutions + 0.2 cc. of 1 in 10 guinea pig complement + 0.2 cc. of 5 per cent red cells.

Serum.	Serum dilution.										
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1,024
Dog A Untreated....	C.	C.	+++	++	++−	+	+	Tr.	Ft. Tr.	0	0
Dog A Exhausted....			No hemolysis.								
Dog B Untreated....	C. (?)	Alm. C.	Alm. C.	++++	++	+−	+−	Tr.	Ft. Tr.	0	0
Dog B Exhausted....			No hemolysis.								

Hemagglutination.—The mixtures were the same as those for hemolysis save that 0.2 cc. of salt solution was substituted for guinea pig complement.

Serum.	Serum dilution.										
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1,024
Dog A Untreated..	C.	C.	C.	C.	+++	+++	++	Tr.	Tr.	0	0
Dog A Exhausted..	++++	+++	++	Tr. (?)	0	0					
Dog B Untreated..	C.	C.	Alm. C.	++++	++++	++	Tr.	0	0	0	0
Dog B Exhausted..	++	Tr.	0	0							

Although some hemagglutination was noted in the mixtures with exhausted serum after they had stood over night, no trace of this was observable when they were first taken from the incubator. Many hemagglutinins, as Landsteiner and Reich[10] first showed, act most strongly at low temperature.

Because of these findings, which showed that the exhaustion of the immune sera with rabbit cells had been practically complete, no similar tests were made of the exhausted normal sera. For the anti-rabbit titer of these sera, when unexhausted, was, relatively speaking, slight.

Pneumococcus Agglutinins.—These were present in the immune sera but were weak, being effective on macroscopic test only in dilutions up to 1 in 16 of each serum. They were found to be unaffected by exhaustion of the sera. The details of the tests need not be given.

In Vivo Tests of the Protective Power of the Exhausted Antipneumococcus Serum.—With the change in plan that determined the use of mice instead of

[10] Landsteiner, K., and Reich, M., *Centr. Bakteriol.*, *1te Abt.*, *Orig.*, 1905, xxxix, 83.

rabbits for the protection experiments, it became unnecessary to make *in vivo* tests as to whether the sera exhausted with red cells had been deprived of their toxicity for the species furnishing the cells; that is to say, for rabbits. The results with exhausted anti-guinea pig serum of far higher original titer, which have already been described, were deemed sufficient on the point, especially since tests showed that the unexhausted dog serum contained not the least nephrotoxin for the rabbit, despite the repeated use of the renal tissue of rabbits as an antigen. The method adopted for the protection experiments with mice was that now familiar from the work of Avery, Chickering, Cole, and Dochez.[11] An 18 hour culture in pneumococcus broth of the Neufeld strain of pneumococcus was used in a series of tenfold dilutions with this broth. Of each culture dilution 0.5 cc. was drawn into a Record syringe, then 0.5 cc. of the serum under test was taken up, and the whole was at once injected into the peritoneal cavity of a 20 gm. mouse. The control sera were handled after the immune sera, so that the former had the advantage of any attenuation in the pneumococcus suspensions which might have occurred during the period of the injections. Last of all, some mice were given a set of control mixtures containing 0.5 cc. of broth instead of a serum.

The animals that died were autopsied promptly and films taken of the peritoneal exudate and heart's blood. These in every case showed the pneumococcus in pure culture. No mouse was put down as surviving until 4 days after injection.

Serum.		Amount of culture.					
		0.000001 cc.	0.00001 cc.	0.0001 cc.	0.001 cc.	0.01 cc.	0.1 cc.
		hrs.	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*
Broth controls		D.* in 26	D. in 28	D. in 32	D. in 20	D. in 16	
Immune A	Unexhausted	Lived.	Lived.	" " 42	" " 26	" " 23	D. in 17
	Exhausted	"	"	Lived.	" " 89	Lived.†	" " 11
Immune B	Unexhausted	"	"	"	Lived.	D. in 17	" " 14
	Exhausted	"	"	"	D. in 32	" " 49	Lived.
Normal a	Unexhausted	D. in 30	D. in 30	D. in 23	" " 17	" " 17	D. in 7½
	Exhausted	" " 31	" " 21	" " 20	" " 16	" " 11	" " 9
Normal b	Unexhausted	" " 27	" " 30	" " 25	" " 22	" " 12	" " 9½
	Exhausted	" " 31	" " 50	" " 22	" " 11	" " 9	" " 9
Normal c	Unexhausted	" " 30	" " 42	" " 19	" " 14	" " 14	" " 7½
	Exhausted	" " 40	" " 28	" " 28	" " 22	" " 12	" " 10

* D. indicates died.

† Slight escape of injected fluid beneath skin.

[11] Avery, O. T., Chickering, H. T., Cole, R., and Dochez, A. R., Acute lobar pneumonia. Prevention and serum treatment, Monograph of The Rockefeller Institute for Medical Research, No. 7, New York, 1917.

Comment.

The experiment shows that the immune dog sera protected mice against about 100 times the amount of pneumococci that was fatal when normal dog serum was employed. The exhaustion of the immune sera with four successive portions of rabbit red cells did not diminish in the least its protective character. The question may be asked, why was such an oblique method used to demonstrate the availability of the exhausted serum? Instead of immunizing dogs with rabbit tissue, exhausting the dog serum with rabbit red cells, and testing protection on mice, might not these latter animals have been employed throughout, to the elimination of rabbits? This was not practicable for several reasons. It would have been difficult to obtain enough sterile normal tissue from mice for the production of a strong anti-mouse serum, and, granting that such a serum could eventually have been elicited, the problem would have arisen of obtaining sufficient mouse red cells for its exhaustion. Undoubtedly the spleens of mice dying of pneumococcus infection would have furnished a powerful antigen, as concerns this organism; but so little of the splenic tissue could have been employed in the immunization, owing to the virulence of the pneumococci therein contained, that it is doubtful whether the serum resulting would have been strongly anti-mouse. And a serum strong in anti-tissue elements was desirable for our type experiments.

Selective Absorption Applied to a Serum Conferring Protection against Poliomyelitis.

As enlarging the general scope of the work, a test was made of whether exhaustion with red cells would deprive the serum of a monkey recovered from poliomyelitis of its protective power against this disease.[12]

The serum of an immune monkey might conceivably be used in the treatment of human beings after exhaustion of its anti-human elements. For this reason the selective absorption was carried out with human cells, though in the ultimate test of protection monkeys were of

[12] The experiment was rendered possible through the cooperation of Dr. Amoss.

necessity employed. The choice of cells was a poor one, because the anti-human elements in monkey serum are extremely weak[13] and are not readily enhanced by tissue injections. The test of the persistence of antipoliomyelitic elements in serum submitted to selective absorption with human cells is in consequence not a drastic one.

Experiment.—A *Macacus rhesus* monkey recently recovered from poliomyelitis, and with severe residual paralyses, was given intravenously on 3 successive days portions of a mixture of defibrinated human blood and an extract in salt solution of human placenta ground with sand. 10 days after the last injection the animal was bled for serum. This on test showed no hemolysin for human red cells and only weak agglutinins. It was exhausted as follows, according to the usual technique.

Mixture.	Hemagglutination.
2.25 cc. of serum + 0.5 cc. of human red cells, incubated 5 min.;	Massive.
and serum transferred to 0.25 cc. of human red cells, incubated 45 min.;	Moderate.
and serum transferred to 0.25 cc. of human red cells, incubated 45 min.	Faint.

The exhaustion was nearly complete, as shown by tests in which one part of serum in graded dilutions + one part of 5 per cent human red blood cells were mixed in Wright's tubes and examined microscopically after 15 minutes at room temperature.

Agglutination.

Serum.	Serum dilution.						
	0	1/2	1/4	1/8	1/16	1/32	1/64
Untreated	C.	−	−	+	+−	Ft. Tr.	0
Exhausted	+	Tr.					

The test of protective power was carried out by Dr. Amoss, who mixed 2 cc. of the exhausted serum with 0.2 cc. of freshly prepared poliomyelitic virus, and injected the whole, after 2 hours incubation, into the cerebrum of a normal *rhesus* monkey. The animal was one of a considerable number receiving an equal amount of the same virus mixed with various sera, so the experiment was well controlled. Eight monkeys were given mixtures of 0.2 cc. of virus + 2 cc. of

[13]Marshall, H. T., *J. Exp. Med.*, 1901–05, vi, 347.

normal human or monkey serum, and all came down with poliomyelitis after from 5 to 7 days and died. The animal receiving exhausted immune serum mixed with virus remained entirely free from the disease.

The indication from this one experiment is clear, that the principle neutralizing the virus of poliomyelitis persists in immune serum exhausted with red cells. Owing to the difficulty of obtaining immune monkeys the work has not been repeated.

SUMMARY.

Attempts to produce antisera in animals to combat specific infections are usually deferred until the cause of the infection has been isolated and grown in pure culture to furnish antigen. It has seemed to us that the fulfillment of these conditions might in some cases be rendered unnecessary through the use of infected tissue itself as an antigen, combined with selective absorption of the antiserum to rid it of elements injurious to the species furnishing the tissue. In order to test this possibility type experiments have been carried out with immune sera effective against known antigens of three different sorts:

1. Sera resulting from the injection of rabbits and a goat with normal guinea pig tissues and a bacterial hemotoxin, the megatheriolysin described by Todd, which hemolyzes guinea pig cells. The sera possessed strong antitoxins for the megatheriolysin but were fatal to guinea pigs. By the method of selective absorption they were rendered innocuous to these animals and were successfully used to protect them from lethal doses of the megatheriolysin.

2. Anti-rabbit dog sera containing antibodies protective against pneumococcus infection. Such sera, subjected to repeated absorption with rabbit red cells, proved capable of protecting mice from pneumococcus infection in exactly the same degree as the unexhausted serum; that is to say, they protected against 100 times the dose of pneumococci that was fatal with normal dog serum.

3. The serum of a monkey recovered from poliomyelitis and repeatedly injected with human red cells and extract of placental tissue. This serum, after selective absorption with human red cells, protected a monkey against an intracerebral dose of poliomyelitic virus

fatal to eight other monkeys given it with normal monkey or human serum.

The results in these instances, purposely chosen for their simplicity, would seem to indicate for the absorption method some usefulness in the study of immunity to infections of unknown cause. In Part II of our paper the method is applied to one such infection; namely, a sarcoma of the fowl engendered by a filterable agent. A general discussion will be found in connection with this portion of the work.

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EXPERIMENTS ON THE PRODUCTION OF SPECIFIC ANTISERA FOR INFECTIONS OF UNKNOWN CAUSE.

II. The Production of a Serum Effective against the Agent Causing a Chicken Sarcoma.

By PEYTON ROUS, M.D., OSWALD H. ROBERTSON, M.D., and JEAN OLIVER, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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The work detailed in Part I of this paper has served to demonstrate the theoretical usefulness of exhausted sera to combat infections, but it presents no instance of a serum actually resulting from the direct immunization of animals by injections of infected tissue. Such an instance is highly desirable. It has been furnished through experiments with a transplantable chicken sarcoma,[1] known in our laboratory as Chicken Tumor I, which has a filterable agent as its cause. The exact nature of the filterable agent is unknown, but its general characters would seem to place it with the microorganisms.[2] The tumor is a typical sarcoma, highly malignant, and as a rule rapidly fatal to fowls developing it after an implantation with neoplastic tissue or inoculation with the Berkefeld filtrate of a tumor suspension. Some individuals are primarily insusceptible, and in some the growth develops slowly, and eventually retrogresses. The latter fail ordinarily to develop a sarcoma when reinoculated. Repeated unsuccessful attempts have been made to demonstrate antibodies in the blood of fowls in which a growth has retrogressed, and to render others immune to the tumor by injections with heated or dried neoplastic tissue.[3] The tumor cannot be transmitted to geese, ducks, pigeons, or mammals; but attempts to develop an antiserum by the immunization of such animals have been blocked through failure to obtain the tumor-

[1] Rous, P., *J. Exp. Med.*, 1910, xii, 696; *J. Am. Med. Assn.*, 1911, lvi, 198.

[2] Rous, P., and Murphy, Jas. B., *J. Am. Med. Assn.*, 1912, lviii, 1938.

[3] Rous, P., and Murphy, Jas. B., *J. Exp. Med.*, 1914, xx, 419.

producing agent in culture. The employment in these alien species of the neoplastic tissue itself as an antigen, or a filtrate from such tissue, elicits, of course, anti-chicken elements in the immunized individual.[4] The method of specific absorption to obtain an antiserum here finds a direct application.

Immunization of Animals.—The blood of fowls carrying the chicken tumor often contains during the last few days of life the causative agent of the disease; and in the sarcomatous tissue the agent is regularly present in large quantity. Both blood and tissue could therefore be used in the immunization, which was desirable in order to insure the production of a strong anti-chicken serum. Chickens moribund with the growth were bled to death under aseptic conditions, the blood was citrated, and the tumor tissue itself was ground with sand and suspended in Locke's solution just prior to injection. As the causative agent of the growth will withstand repeated freezing and thawing and retains its activity for a long period at low temperature, the material often was kept in the frozen state for days or weeks prior to use.

The first attempts to obtain an antiserum were made with rabbits. A number of these animals were injected intravenously on 3 successive days with a tumor extract in salt solution, and thereafter intraperitoneally every 6 days with citrated chicken blood and a suspension of tumor tissue. But though the serum soon acquired a high content of chicken hemolysins and hemagglutinins it had not the least neutralizing effect on the tumor-causing agent present in Berkefeld filtrates of suspensions of the sarcoma tissue. For this reason work with rabbits was at length discontinued.

Implanted bits of the chicken sarcoma perish at once in mammals, whereas in ducks and pigeons they grow for some days before retrogressing and may form quite large nodules. It seemed from this fact not improbable that birds would prove more favorable than rabbits as producers of tumor antibodies, owing to what might be considered as a partial susceptibility on their part to the neoplastic disease. For Flexner and his associates[5] have shown that in the case of poliomyelitis an immune serum is obtained only in species susceptible to the infection. Geese were used, therefore, in the further attempts to obtain an antiserum. Their immunization was carried out as follows:

Goose A received three intravenous injections on successive days of mixed tumor suspension and citrated blood from fowls moribund of the growth, followed thereafter every 6 or 7 days by intraperitoneal injections of the same material. Goose B was given the same sort of material, but only into the peritoneal cavity. From time to time both birds were bled from a wing vein and the sera compared

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[4] Bailey encountered this difficulty in experiments on complement fixation with the serum of pigeons inoculated with the growth (Bailey, C. H., *Med. Rec.*, 1915, lxxxviii, 403).

[5] Personal communication from Dr. Flexner.

in neutralizing properties with those of three normal geese. During a long period of immunization no differences could be noted, but on the contrary a similarity in the five sera so entire as to indicate that the blood of one normal goose is just like that of any other in its effect, or rather lack of effect, on the tumor-producing agent. At length, as will be seen from the experiments now to be cited, an immune principle became demonstrable in the serum of the injected birds.

Experiment 1.—The immunized geese, A and B, were bled for serum 84 and 66 days respectively after immunization was begun, and 9 days after the last injection of tumor material. Goose A had received three intravenous and ten intraperitoneal injections, while Goose B had but nine injections, all intraperitoneal. Two normal geese (a and b) were bled at the same time and to an equal amount; namely, 75 cc. The sera were inactivated as usual, and all were submitted to absorption with similar portions of washed chicken red cells, as follows:

Mixture.	Hemagglutination.	
	Immune sera.	Normal sera.
25 cc. of goose serum + 5.2 cc. of chicken red blood cells incubated 1 hr. and	+	0
serum transferred to 4 cc. of chicken red blood cells, incubated 1 hr.	Tr.	0

Cultures taken after the second absorption proved sterile. Preliminary tests showed that the untreated immune serum failed to hemolyze chicken cells when chicken serum was used as complement, whereas these were rapidly destroyed in the presence of guinea pig complement. Consequently the latter was used in the titrations that follow.

Anti-Chicken Titer of the Sera. Hemolysis.—0.2 cc. of inactivated serum in graded dilutions + 0.2 cc. of 1 in 10 guinea pig complement + 0.2 cc. of 5 per cent guinea pig red cells. Incubation and ice box was for 2 hrs. at 38°C. and readings were made after the tubes had stood in the ice box over night.

Serum.	Serum dilution.								Complement + red cells + 0.2 cc. of salt solution.
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	
Untreated immune. A	C.	C.	C.	Alm. C.	Alm. C.	+++	Tr.	Ft. Tr.	0
Untreated immune. B	++++	+++	++	+	+−	Tr.	Ft. Tr.	0	
Untreated normal. a	Tr.	Ft. Tr.	0	0	0	0			
Untreated normal. b	"	" "	Ft. Tr.	0	0	0			
Exhausted sera	No hemolysis by any.								

The selective absorption had completely deprived the immune sera of their relatively strong hemolysin.

Hemagglutination.—This was read in mixtures similar to the foregoing but containing chicken serum (1 in 10) as complement. None of the tubes showed any hemolysis with this complement, but those containing undiluted immune goose serum exhibited a slight hemagglutination. None of the exhausted sera agglutinated chicken cells in the least.

Precipitation.—The normal sera contained no precipitin, but a weak one was present in the immune sera. It was active against dilutions of chicken serum up to, and including, 1 in 40.

In Vivo Tests of Neutralization.—The exhausted sera only were used in neutralization tests. For this purpose mixtures were made of the sera with a Berkefeld filtrate containing the tumor-producing agent, and these after incubation were injected into fowls. In some early experiments mixtures of the filtrate with isotonic saline or Locke's solution were employed as controls, but it was found that they soon lost their tumor-producing activity when incubated, whereas this was retained in mixtures with normal goose serum, either untreated or exhausted. Consequently in the present experiment, as in others to be detailed, the mixtures with normal sera constitute the controls.

The tumor filtrate was prepared by grinding fresh neoplastic tissue with sand, making a thin suspension in Locke's solution, shaking, centrifuging, and passing the clear fluid through one or another of several Berkefeld filters (N). Several filters were used to ensure an active filtrate, since the tumor-producing agent is held back by many of the finer Berkefeld candles, and all the filtrates were united. Now two mixtures were made with the sera: (1) 15 cc. of each exhausted serum + 7 cc. of filtrate; (2) 7 cc. of each exhausted serum + 2 cc. of filtrate. These were incubated for 2 hours at 38°C. They remained water-clear. 1 cc. of a suspension of sterile diatomaceous earth was added to each, and portions of all were injected into each of a number of chickens. The mixtures with immune sera were injected first so that any possible advantage as regards attenuation of the virus during incubation, or neutralization of it, might lie with the mixtures containing the normal serum. Diatomaceous earth was added because, through the tissue injury it causes, the production of tumors by a filtrate is rendered much more certain.[6]

The ten chickens inoculated received 3 cc. of each mixture, into the pectoral muscles and the muscles of the upper wings respectively. Usually the tumor grows fastest and becomes largest in the pectoral muscles, and for this reason the injection site for the mixtures was varied from bird to bird; but in the experiment now under consideration no favoring influence of the pectoral situation

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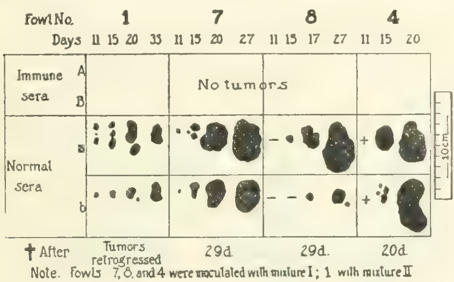
[6] Rous, P., Murphy, Jas. B., and Tytler, W. H., *J. Am. Med. Assn.*, 1912, lviii, 1751.

was to be seen. The growths did not attain a very large size before death ensued from metastases.

Clear-cut findings were obtained, as Text-fig. 1 shows. Only four of the ten fowls developed tumors. In them growths failed to appear where the mixtures of immune sera and filtrate had been injected, whereas at the control sites large ones developed.

Experiment 2.—The same general plan was followed as in the preceding experiment, but the immunized geese had now received two additional intraperitoneal injections. Bleeding for serum was done 121 and 103 days respectively from the time immunization of the birds was started, and 7 days after the last



TEXT-FIG. 1. The tumors in four fowls receiving intramuscular injections of mixtures of tumor filtrate with immune and normal goose sera respectively.

injection. The sera of three normal geese, a, b, and c, were used in control. Selective absorption was carried out as usual.

30 cc. of goose serum + 5.8 cc. of chicken red cells incubated 1 hr. and
serum transferred to 2.9 " " " " " " 1 " "
" " " 2.8 " " " " " " 1 "

Cultures taken after the last absorption proved sterile.

Anti-Chicken Titer of the Sera. Hemolysis.—0.2 cc. of inactivated serum in graded dilutions + 0.2 cc. of 1 in 10 guinea pig complement + 0.2 cc. of 5 per cent chicken red cells.

Serum.		Serum dilution.								
		0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Untreated immune.	A......	C.	C.	C.	Alm. C.	++	+	Tr.	0(?)	0
	B......	Alm. C.	Alm. C.	+++	++	+	Ft. Tr.	0	0	0
Untreated normal.	a......	No hemolysis.								
	b......	+−	0	0	0	0				
	c......	+++	++	+	0					
Exhausted sera......		No hemolysis by any.								

Hemagglutination.—0.2 cc. of inactivated serum in graded dilutions + 0.2 cc. of 5 per cent chicken red cells + 0.2 cc. of salt solution.

Serum.		Serum dilution.				
		0	1/2	1/4	1/8	1/16
Untreated immune.	A..............	+	+	+	Tr.	0
	B..............	++	Tr.	0	0	0

With the exhausted normal and immune sera, as well as the untreated normal sera, no agglutination was obtained.

Precipitation.—There was no precipitin in the normal sera, but one was present in that from both immune geese. It was effective in mixtures of equal parts of the undiluted goose serum with dilutions of chicken serum up to and including 1 in 40 for Goose A and 1 in 20 for Goose B. The titer was little if at all diminished by the absorption with red cells.

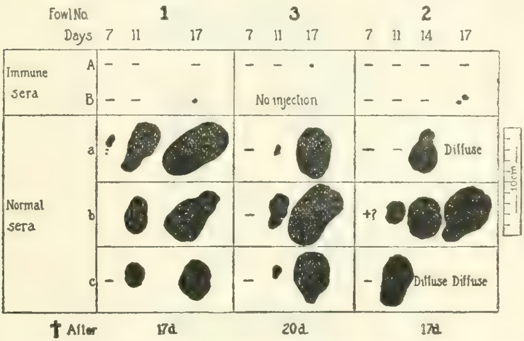
In Vivo Tests of Neutralization.—A Berkefeld filtrate of a tumor extract was prepared by the method already described, and three mixtures were made of it with the exhausted sera, both normal and immune.

Proportion X: 7.5 cc. of serum + 2 cc. of filtrate.
Proportion Y: 12 cc. of serum + 6 cc. of filtrate.
Proportion Z: 7 cc. of serum + 7 cc. of filtrate.

Incubation was for 2 hours at 37°C. No precipitation or clouding occurred. A suspension of diatomaceous earth was now added to each mixture in the amount of one-tenth its volume, and the injection of fowls was forthwith begun. Fifteen fowls were used, and all save four received 3 cc. of each mixture, the site of injection being varied. The four fowls mentioned were not given the mixture con-

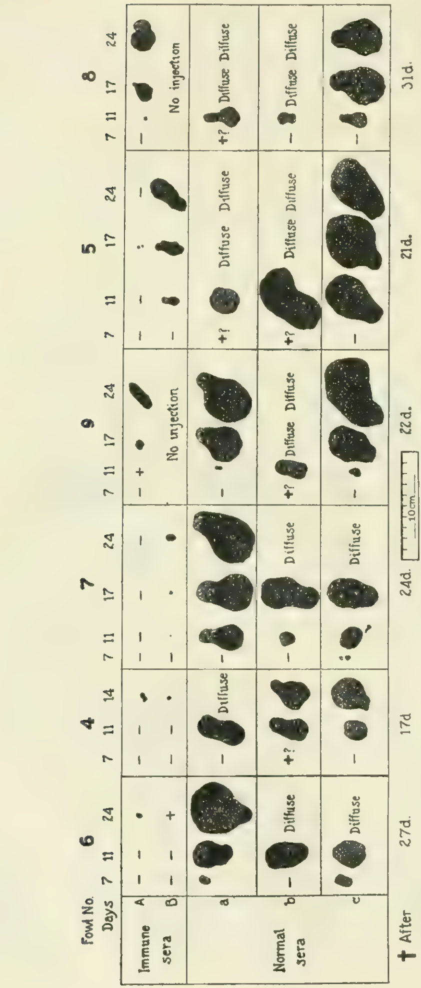
taining the serum of Immune Goose B. The injections were made into the upper wing, upper leg, and pectoral muscles. As Text-figs. 2, 3, and 4 show, large growths rapidly developed where the control mixtures had been placed, whereas none, or only slowly growing ones, were caused by the mixtures containing immune serum.

The neutralizing effect on the tumor-producing agent of the exhausted serum of geese immunized with tumor tissue is clearly shown by these protocols. The agent was especially active in the filtrate used in Experiment 2, as shown by the fact that every one of the fifteen inoculated fowls developed tumors—an occurrence unparalleled in our records. The immune serum completely prevented

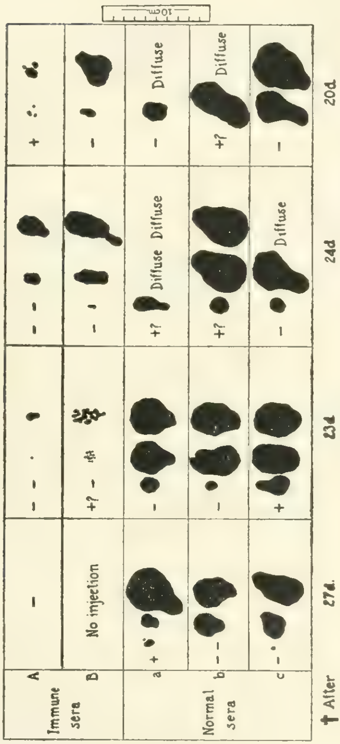


TEXT-FIG. 2. The tumors developing in three fowls receiving mixtures in Proportion X.

tumors at only three injection sites in these fowls, though its protective influence was manifest wherever it had been injected. Very large tumors resulted from all three normal serum mixtures, whence it may be inferred that even the smallest amount of filtrate present in any one, namely that of Proportion X (about 0.66 cc. of filtrate per fowl), contained what might be termed a maximum tumor-producing dose of causative agent. More than twice this amount (1.5 cc. in Proportion Z) yielded tumors that were no larger and grew no more rapidly. The test of the neutralizing power of the immune sera was evidently a severe one in this experiment. In Experiment 1 the filtrate was far less active, as shown by the large proportion of nega-



TEXT-FIG. 3. Tumors in six fowls receiving mixtures in Proportion Y.



TEXT-FIG. 4. Tumors in four fowls receiving mixtures in Proportion Z.

tive fowls (six out of the ten inoculated) and the slow course of the tumors that appeared. Here the neutralization of the tumor-producing agent by the exhausted serum of the immunized geese was complete.

To what is the neutralization referable,—unabsorbed remnants of chicken antibodies? This possibility may be tested by determining whether chicken antibodies as such are able to neutralize the tumor-producing agent. The results with the sera of immunized rabbits gain importance in this connection. For the rabbit sera, while strongly anti-chicken—many times more so than the goose sera—had not the least neutralizing effect on a tumor filtrate.

Experiment 3.—A rabbit was given three intravenous injections on successive days of a saline extract of chicken tumor, followed at 6 day intervals by eight intraperitoneal inoculations of a mixture of tumor suspension and citrated blood from fowls moribund of the growth. 8 days after the last injection the animal was bled to death, and its inactivated serum was compared in neutralizing power with that of a normal rabbit. Selective absorption of both was carried out as usual.

Mixture.	Hemagglutination.
15.5 cc. of rabbit serum + 4 cc. of chicken red blood cells, incubated 1 hr. and	Marked.
serum transferred to 4 cc. of chicken red blood cells, incubated 1 hr.......	0

Anti-Chicken Titer of the Sera. Hemolysis.—0.25 cc. of inactivated serum in graded dilutions + 0.25 cc. of 1 in 10 guinea pig complement + 0.25 cc. of chicken red cells.

Immune serum.	Serum dilution.															Guinea pig complement + salt solution + red cells.
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1,024	1/2,048	1/4,096	1/8,192	1/16,384	
Untreated..	C.	C.	C.	C.	C.	C.(?)	Alm. C.	+++	+++	+++	+	±	Tr.	Tr.	Ft. Tr.	0
Exhausted..	+	+	±	±	Tr.	Tr.	Tr.	F. test.	0	0						

Exhaustion was in this instance only approximately complete.

Hemagglutination.—The mixtures were the same as those above except that 0.25 cc. of 0.9 per cent salt solution was substituted for guinea pig complement.

Immune serum.	Serum dilution.									
	0	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Untreated.....	+++	Alm. C.	Alm. C.	C.	C.	++	++	Tr.	—	0
Exhausted....	No agglutination.									

The normal rabbit serum destined to be used in control of the *in vivo* work caused only the slightest hemolysis of chicken cells and no agglutination, when tested prior to its absorption. Thereafter it did not affect the cells at all.

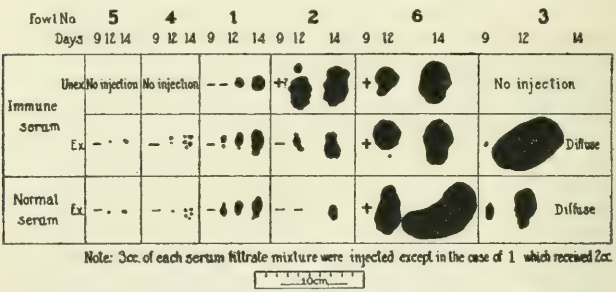
Precipitation.—The normal rabbit serum was entirely inactive, but that of the immunized animal caused precipitation when incubated with equal parts of chicken serum diluted up to and including 1 in 2,560.

In Vivo Tests of Neutralization.—Three serum specimens were used—normal and immune serum, exhausted as above, and untreated immune serum. A Berkefeld filtrate containing the tumor-producing agent was prepared as usual and mixed with the rabbit sera in the proportion of 6 cc. of filtrate to 12 cc. of serum. Incubation at 38°C. was carried on for 2 hours, cultures were taken, portions of a suspension of diatomaceous earth in salt solution were added to each mixture (0.7 cc. for every 20 cc. of mixture), and injections were made of 3 cc. into five fowls and of 2 cc. into a sixth. In the mixtures with immune serum a floccular precipitate had come down which was distributed by shaking prior to the injections. The sites of injection were varied, as usual. The cultures of the injection fluids were negative after 2 days. Tumors developed in all the fowls, as Text-fig. 5 shows.

The test of the neutralizing power of the rabbit sera was in this case not a severe one. For the late appearance and slow growth of the control tumors clearly showed that no excess of tumor-producing agent was present in the mixtures. Yet there is not the slightest indication of any effect upon the agent of the immune serum, even when it had not been exhausted and was very strong in chicken hemolysin, agglutinin, and precipitin. Said serum had exactly the same effect as serum from a normal rabbit, which contained only the weakest antibodies for the chicken. A floccular precipitation occurred in the mixtures of filtrate and immune serum, but so slowly that it can scarcely have afforded to the tumor-producing agent much protec-

tion from other serum antibodies; and only complete protection by it would explain the results in the inoculated fowls.

This experiment would seem to prove that the neutralization of the tumor-producing agent by the serum of immunized geese is not due to antibodies directed against chicken tissue as such. Such antibodies —or at least those elicited in the immunization of rabbits—fail entirely to injure the tumor-producing agent, even when they are very strong. In view of these facts, the conclusion seems justified that the neutralization of the agent causing a chicken tumor by the serum



TEXT-FIG. 5. Tumors arising in six fowls injected with tumor filtrate mixed with normal and immune rabbit serum.

of geese repeatedly injected with the tumor tissue is not the result of the action of antibodies directed against the chicken tissue as such, but is due to others specific for the tumor-producing agent. These are retained by goose serum exhausted with chicken red cells.

DISCUSSION.

The selective absorption of tissue antibodies has been applied thus far to four immune sera of widely different properties (see Part I of this paper), with success in each instance. There is no doubt that by the method sera can be deprived of antibodies immediately injurious to the animal organism while retaining those directed against an infectious agent or its products. Applications of the principle in the treatment of disease at once suggest themselves. But many points

must be determined before any practical therapeutic venture is warranted.

First, the late or latent effects on the animal body of exhausted serum must be closely studied. Serum precipitins are not removed with hemolysins and hemagglutinins during the process of exhaustion with red cells. What then is the effect of a specific precipitin acting *in vivo* on an animal of the species against which it is directed? We have been unable to find in the literature a conclusive answer to this obvious question. The controversy over the relation of precipitation to anaphylaxis has resulted in a multitude of *in vivo* experiments, but these have been carried out almost exclusively by introducing precipitin and precipitinogen into animals to which both are alien, or by injecting a serum precipitinogen into an organism that possesses, or will develop, a precipitin for it. Uhlenhuth and Haendel[7] and Doerr and Moldovan[8] have claimed that anti-guinea pig rabbit serum of high precipitin titer is toxic to guinea pigs when injected intravenously; but these authors made no attempt to absorb from the serum the hemolysins and agglutinins present in it and undoubtedly capable of harmful effects. Their work has not been followed up. We plan to do this.

It seems not unlikely that an antiserum resulting from injections of tissues, especially tissues other than blood, will contain elements of possible harm besides hemolysins, hemagglutinins, and precipitins. Here one is confronted with the problem of the specificity of cytotoxins, so long and indecisively debated. Fortunately we are concerned with a single aspect of this problem; namely, that of whether specific cytotoxins, assuming that they exist for the generality of organs—a large assumption—can be removed from serum by its exhaustion with red corpuscles. For should they not be so removable it may be necessary to exhaust a serum with the same kind of tissue employed in the immunization, a matter of much practical difficulty. Experiments on the point with a specific cytotoxic serum, so called, have been begun.

Theoretically the most important use of exhausted sera lies in the treatment of infections of unknown cause. And with each such in-

[7] Uhlenhuth and Haendel, *Z. Immunitätsforsch., Orig.*, 1910, iv, 761.

[8] Doerr, R., and Moldovan, J., *Z. Immunitätsforsch., Orig.*, 1910, vii, 223.

fection two fundamental points would have of necessity to be determined. They are (1) whether the infected tissue will suffice as a practical antigen, and (2) whether the antibodies useful against the infection or its products will survive the serum's exhaustion of antibodies injurious for tissue. The microorganisms in infected tissue employed as antigen will be in many instances in the highest state of pathogenicity. There are advantages to this, but also drawbacks. If the animals to be immunized are themselves susceptible to the infection much less fresh tissue antigen can be employed than of one attenuated by culture or in another way. The dosage of antigen will also be difficult to regulate. Both these obstacles were encountered in Part I of the present work, during our attempts to immunize dogs by injecting them with the blood of rabbits dying of pneumococcus septicemia. So large a percentage of the dogs died that resort was had at length to an antigen of normal tissues and pneumococcus cultures injected separately. The conditions would be much more favorable to successful immunization in the case of infections only slightly pathogenic to the animals employed for immunization. Here tissue containing the infective agent in most virulent form would have great advantages and not improbably decisive ones in the case of cultivable agents that lose their pathogenicity, and incidentally their usefulness as antigen, when grown *in vitro*. Furthermore, it is conceivable that with an agent in highly virulent form so little of the tissue containing it might in certain instances be required as antigen that the serum's titer in elements injurious for tissue would be slight, and the exhaustion in consequence a relatively simple matter.

Little can at this time be said on the persistence of desirable antibodies in an exhausted serum, further than that our experiments make this seem probable in most instances, as do also the observations of others who have used the method of selective absorption to a different end; namely, to demonstrate the specificity of antibodies.[9] Should it become necessary to exhaust a serum of precipitin by means of precipitation in order to render it harmless *in vivo*, even this, it

[9] A noteworthy demonstration of the possibilities of the method is to be found in the work of Todd, C., and White, R. G., *Proc. Roy. Soc. London, Series B*, 1910, lxxxii, 416. By the selective absorption of induced isohemolysins these authors were enabled to recognize the red corpuscles of individual oxen

would seem, might be done without, in most instances, removing the antibodies directed against an infectious agent. For Gay and Stone[10] have made many attempts to bring down such elements in a serum precipitate, but without success.

Although the use of exhausted serum in the treatment of infectious diseases is at present but a distant possibility, there lies open a field for its immediate employment. Through the method of absorption much may be learnt regarding serum immunity to animal diseases—as witness the case of the chicken sarcoma,—and to human infections of unknown cause that are transmissible to animals. For the tissues of infected animals will furnish a ready antigen for experimental purposes, while normal individuals of the same species can be used as test objects to determine whether the exhausted sera resulting from immunization possess any protective power. A concrete illustration of such a possibility is afforded by some recent work of Nicolle and Blaizot.[11] These authors state that they have produced an effective antityphus serum in donkeys by injection with the spleens of guinea pigs dying of the disease. The serum is intended for use in human beings, but they find that with it guinea pigs can be cured of typhus, though the serum is so toxic for such animals that it can be given only in small quantities, which hinders the tests. It would have been interesting to deprive the serum of this toxicity by selective absorption with guinea pig cells, with a view to a more striking demonstration of its antityphus power.

SUMMARY.

By the method of selective absorption with tissue, protective serum antibodies have been demonstrated in the case of an infection of unknown cause; namely, a chicken sarcoma transmitted by a filterable agent. Geese were repeatedly injected with the finely ground sarcoma and with blood from fowls moribund of it; and their sera acquired the power to prevent the tumor-producing agent from causing growths. That this was not due to antibodies elicited by the chicken tissue as such was shown by exhaustion of the goose sera with chicken

[10] Gay, F. P., and Stone, R. L., *J. Immunol.*, 1916, i, 83.

[11] Nicolle, C., and Blaizot, L., *Ann. Inst. Pasteur*, 1916, xxx, 446.

red cells, a step which had not the least effect on the tumor-preventing power, and also by experiments with rabbits immunized as were the geese. These animals developed strong chicken antibodies in their sera which failed nevertheless to affect the tumor-producing agent.

Serum immunity to the chicken sarcoma is weak at best; and in the case of some other infections of unknown cause, more striking results may be anticipated from the method of selective absorption. It is even conceivable that by its means sera of therapeutic usefulness may become available. But much remains to be settled as regards the dangers of exhausted sera and the limitations of the method. Fortunately there exists an immediate field for the latter in laboratory studies on the nature of immunity to infections of which the cause has not been recognized.

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EXPERIENCES WITH A RECENT EPIDEMIC OF MENINGOCOCCIC MENINGITIS AMONG A CHINESE CIVIL POPULATION.

By PETER K. OLITSKY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

During the past spring and early summer (1918) a study was made of an epidemic of meningococcic meningitis occurring among a civil population in a district in South China. Some of the results of the investigation have proved of sufficient interest to warrant their somewhat detailed description, in the hope that they may be of value to the medical officers of the Army and Navy combating epidemic meningitis.

General Description.

The beginning of the epidemic is obscure. An epidemic of meningococcic meningitis heretofore among the Southern Chinese is practically unknown. Hence, when the first cases occurred, the proper diagnosis was not made. It was not until the second week in February that cases which were regarded previously as hemorrhagic smallpox were definitely diagnosed as epidemic meningitis. From that time until the first of June there were reported officially, 1,041 cases; but it is estimated that, including the "missed" cases, the actual number was nearer 2,500. Among the cases reported the mortality was 85 per cent. The epidemic continued until the first week of July; the largest number of cases occurred in March.

The source of infection is likewise indefinite. Although no epidemics have occurred in this district, yet it is reasonable to assume the occurrence of sporadic cases now and then. Indeed, there is a case on record which appeared five years ago. Again, in the Philippine Islands, which are on a direct trade route, there were reported about three years ago seventy cases, of which some yielded pure spinal cultures of the meningococcus. Furthermore, this district is a great

gateway to the southern part of the Orient, and enormous numbers of persons pass through it; undoubtedly some are carriers of meningococci.

The locality has points of interest with reference to epidemic meningitis. In the first place, it is situated in the subtropical zone bordering on the tropical. In view of the extent of the epidemic, it becomes problematical whether this disease is one of the temperate zone. In the second place, the district is greatly overcrowded,[1] and in some quarters there exist living conditions quite analogous to those found in an overcrowded camp. Again, the population comprises for the most part poor people, passively resisting western methods, so that the epidemic was disseminated thickly on a good soil.

Observations on Treatment.

The following observations were made at a local hospital where none but Chinese were received. They are based on a total number of 417 patients. At this point it may be stated that in certain sections of China conservatism is still very strong and modern medicine is not favorably entertained. Hence the following statistics offer a sad but interesting study:

One hundred and four patients received neither serum treatment nor lumbar puncture. Of these, 84.6 per cent. died. This mortality corresponds with the general death rate for the disease throughout the district.

In 346 patients lumbar puncture was made from once to five times. Of these 54.1 per cent. died.

In 71 patients, lumbar puncture combined with the spinal injection of antimeningococcic serums having a low antibody content, was made. Of these, 45 per cent. died. These serums were on hand before my arrival.

(Taking at random 10 spinal cultures isolated from local cases, one serum agglutinated 5 of them, but 3 other specimens agglutinated

1. The area of the district is 390 square miles. The official population is 535,100 and the population to the square mile 1,372. But 300,000 of these Chinese live in a small section of this area—a section about 3 miles long and one-half mile wide.

from 2 to 3, usually in dilutions of 1:50. Flexner serum used as a control agglutinated 56 out of 59 similar cultures and usually in dilutions of 1:800.)

Several conclusions may be drawn from these records. First, the mortality is appalling when no treatment is given. Second, spinal tapping by itself, while showing an improvement in the death rate, is still far from a satisfactory therapeutic procedure. Third, the injection of a serum poor in quality offers only slightly better hope. Lastly, a serum low in agglutinin content is apt to be therapeutically ineffective.

When these methods of treatment are compared with the results recently obtained in civil and military practice by proper antimeningococcic serotherapy, it is certain that the employment of the latter is indicated at all times.

Blood Culture Studies.

At present an extensive literature is being reported on the appearance of the meningococcus in the circulating blood. It is stated that the organism appears very early in the course of the disease. On this account the following blood cultures should prove of interest. Owing to certain difficulties no selection of cases could be made, but in this series of ten cases it happened that all were moribund, and in some instances the blood culture was made in the agonal period.

The blood cultures were made by inoculating from 2 to 3 c.c. of whole blood into each of two flasks containing 100 c.c. of veal infusion, 1 per cent. dextrose broth of 0.7 + acidity (phenolphthalein). Besides these flasks, about four or five plates of dextrose agar were poured, each containing 2 c.c. of blood. None of the plates was positive; a positive result was usually indicated by growth in one or both flasks. All cultures thus obtained were tested for type and corresponded with the spinal strain—the parameningococcus in all instances, as I will describe later.

Case 1, from whose spinal fluid parameningococci were isolated, was cultured on the fourth day of illness. The patient died two days later. The blood culture was negative.

Cases 2, 5, 6, 8 and 9 yielded no growth from the spinal fluids (except Case 2), but the appearance of the fluids was typical and they

contained many gram-negative diplococci, intracellular and extracellular.

The five patients died two days after obtaining the culture, which was taken on the fifth day of illness, except in Case 6, when it was taken on the second day of illness. The cultures were negative in all cases.

In Cases 3 and 4 the patients were comatose on the fifth day of illness, when the culture was taken. They died the following day. The blood as well as the spinal fluid showed a growth of meningococci.

Cases 7 and 10 were similar in detail to Case 3, except that there was no coma or petechial rash, but death occurred the day following the taking of the culture, which was positive.

From these cases one observes that of the ten patients, four yielded positive blood cultures, but of the latter all died one day later. It is difficult to interpret the small number of findings; and the evidence is too scant to indicate whether the infection was primary or secondary in the blood in these cases. The specimens were taken antemortem and could be explained as antemortem meningococcal invasions of the blood. However, the results served to emphasize the desirability of intravenous therapy.

The Prevailing Type of Meningococcus among Patients.

Spinal cultures in a pure state were obtained from 60 patients. Fifty-nine of the patients were examined at the height of the epidemic. Of these, 56 were para types and 3 irregular para types. The para type when tested corresponds with Type I of Gordon's (English) classification; the normal, with Type II; and the irregular para possibly with Type III. The para type agglutinations ranged from 1:50 to 1:800; the irregular para types showed the reactions in Table 1.

From this we conclude that during the height of the epidemic almost 95 per cent. of the patients were infected by the same type of meningococcus, the para type.

The remaining case of the sixty patients examined occurred during July when the epidemic was on the wane and the incidence of the disease in a given section was relatively infrequent. It was re-

markable that this patient should yield a normal or regular type meningococcus.

In this connection it is interesting to note that two of the cultures isolated three years previously in the Philippine epidemic were tested and found to be para types. Also an epidemic of meningitis in a city along the trade route from this district and occurring somewhat later, yielded nineteen para types out of twenty spinal cultures isolated. The inference is that during the height of the epidemic almost all the patients were infected with one type, the parameningococcus.

TABLE 1.

Reactions Shown by Irregular Para Types.

Culture Number	Normal Type Immune Serum	Para Type Immune Serum	Polyvalent Serum
021	1:50	1:400	1:800
030	1:100	1:400	1:400
052	1:50	1:200	1:200

Bacteriologic Studies on Carriers.

(*a*). *Contact Carriers.*—No extensive work could be done on contact carriers at the height of the epidemic, as I arrived at the time of its subsidence. I shall, however, discuss the results obtained then by the local bacteriologists who made several cultures.

These cultures were made as routine examinations of contacts with patients for the presence of the meningococcus in the nasopharynx. It appears that the work was very carefully done; only a few cultures were examined at a time and all plate cultures contaminated with saliva were discarded. The criteria for the recognition of the meningococcus were (1) typical colony morphology, (2) absence of pigment, (3) acid production in dextrose and maltose and not in saccharose medium, (4) typical morphology by Gram's stain, (5) ready emulsification of the growth of subcultures. No agglutination tests were made, however. Sheep serum water (1 part serum to 2 parts water) 20 per cent. agar was used for the plate cultures.

Examining once only contacts of several patients, it was found that:

71 Europeans	yielded	7 carriers (9.8 per cent.)
133 Chinese	yielded	9 carriers (6.7 per cent.)
204 examined	yielded	16 carriers (7.8 per cent.)

The ratio of incidence of meningitis among the Europeans to the white population of the district was as 1 is to 1,250; in the case of the Chinese to the colored population as 1 is to 212 (if 2,500 is used as the basis for the number of cases). These cultures were taken at the height of the epidemic; the number of contact carriers in both series is lower than is usually the case.

One deduction, however, can be made, namely, that the carriers were more prevalent among the white population, notwithstanding the fact that very few of the latter were afflicted (4 to 2,500 of Chinese).

(*b*). *Noncontact Carriers*.—As no opportunity presented itself to continue the study of contact carriers, my attention was paid to a class which may be regarded only nominally as noncontact carriers. In the event of an epidemic of so great an extent as this one, a group can hardly be chosen as a representative noncontact class. It was finally decided that a local jail, in which no case of epidemic meningitis developed throughout the epidemic, would offer a suitable source for the determination of the number of carriers for comparative study.

In interpreting the results, these factors should be considered: The prisoners were in the main previous residents of the district. There was a daily average of 600 inmates. The length of confinement before the cultures were taken varied from a few days to several years. The prisoners were kept isolated in individual, well ventilated cells; there was no overcrowding. In brief, the sanitary conditions in the jail were better than in a great part of the Chinese community.

The following cultures were made on unlaked sheep blood, veal infusion agar with 1 per cent. dextrose (defibrinated blood 1 part, agar 15 parts). The cultures were taken from the nasopharynx with a West swab, care being taken to avoid contamination with saliva. The plates, each person's culture on a single plate, were incubated immediately after inoculation.

The total number of Chinese prisoners swabbed was.... 151

Plate cultures contaminated with saliva and therefore discarded were.................................. 21

A. Negative Cultures.

1. Plate cultures showing no suspicious colonies... 74
2. Those showing suspicious colonies which failed to grow characteristically.................. 19
3. Those showing suspicious colonies which were proven negative by agglutination ("flavus agglutination")............................ 3
4. Those showing suspicious colonies which subsequently grew at room temperature.......... 2

Thus making a total of 98 negative cultures.

B. Positive Cultures.

1. Plate cultures showing typical colonies, consisting of gram-negative diplococci of characteristic morphology, but which failed to grow on subculture. There were usually two or three colonies transplanted. On the basis of previous experience it was thought proper to include these with the positive cultures. They were as shown in Table 2.

TABLE 2.

Plate Cultures Showing Typical Colonies.

Serial Number	Age (Years)	Time in Jail	Degree of Infection*
7	20	1 mo.	±
46	25	2 mos. 9 days	+
70	30	2 mos.	±
97	27	1 mo 5 days	±
132	28	5 mos. 14 days	±

* The term "degree of infection" is used to signify the relative number of colonies in the plate cultures. This gives an indication as to the extent of the infection of the nasopharynx. Hence ± signifies an occasional colony; +, few colonies; ++, numerous colonies of meningococci, but other organisms predominating; +++, meningococcus colonies predominating; and ++++, a pure culture of the meningococcus.

2. Plate cultures showing typical colonies consisting of gram-negative diplococci of characteristic morphology; producing acid in dextrose and maltose, but not in saccharose medium; showing no growth

at room temperature, but a typical growth on subculture (ready emulsification, no pigment, mucoid, etc.); showing no agglutination in saline or normal horse serum (1: 50) controls, and no agglutination with type or polyvalent antimeningococcic serum (Table 3).

TABLE 3.

Plate Cultures Showing Inagglutinable Cultures.

Serial Number	Age (Years)	Time in Jail	Degree of Infection
56	31	2 mos. 7 days	++
75	27	14 days	+
76	30	11 days	++
100	22	2 mos. 7 days	+
124	34	3 yrs. 5 mos.	+

3. Plate cultures showing colonies having the characteristics mentioned under 2, but possessing definite agglutination reactions with polyvalent or type serum or both (Table 4).

4. To the last mentioned may be added plate cultures yielding colonies indistinguishable from spinal strains but having no definite type reactions and showing a definite sedimentation along with a slight agglutination with Flexner's serum, but not with normal horse or type serum. As they all reacted similarly, these cultures were placed in one group, as given in Table 5.

Summarizing the results of the positive cultures, we find that there were:

Normal types (corresponding to Type II of the English classification)	11
Para type (corresponding to Type I)	1
Irregular normal type	1
Irregular para type	2
Irregular (no agglutination with normal or para only with polyvalent serum).	7
Inagglutinable types	5
Cultures impossible to agglutinate as no growth was obtained	5
Total	32

Therefore, notwithstanding the fact that no cases occurred in the jail, we find in the series of prisoners examined that 24.6 per cent. harbored meningococcus-like organisms in the nasopharynx.

TABLE 4.

Colonies from Jail Controls Possessing Definite Agglutination Reactions.

Serial No.	Age (Years)	Time in Jail	Degree of Infection	No. of Colonies Exam.	Agglutination Reactions (Dilutions of 1:50 and 1:100)*	Type
1	53	1 yr. 4 mos.	±	1	Poly., ++; normal, ++; para, 0	Normal
20	21	1 mo. 6 days	±	2	Poly., ++; normal, 1:50, +; para, ++	Irregular para
21	34	1 mo. 29 days	++	1	Poly., ++; normal, ++; para, 0	Normal
22	29	11 days	+++	1	Poly., ++; normal, 1:50, +; para, ++	Irregular para
28	27	3 mos.	+	1	Poly., ++; normal, 0; para, 0	Irregular
50	23	1 mo. 27 days	±	1	Poly., ++; normal, ++; para, 0	Normal
82	20	17 days	±	1	Poly., ++; normal, ++; para, 0	Normal
84	46	7 days	±	2	Poly., ++; normal, ++; para, 0	Normal
88	55	1 mo. 9 days	+	2	Poly., ++; normal, ++; para, 0	Normal
89	34	1 mo. 6 days	+++	2	Poly., ++; normal, ++; para, 1:50, +	Irregular normal
102	39	19 days	±	1	Poly., ++; normal, 0; para, 0	Irregular
112	30	29 days	±	1	Poly., ++; normal, 0; para, ++	Para
117	20	1 mo. 14 days	++	1	Poly., ++; normal, 0; para, 0	Irregular

* ++ indicates complete agglutination in a dilution of 1: 100; +, incomplete agglutination in the dilution stated.

TABLE 4—*Continued.*

Serial No.	Age (Years)	Time in Jail	Degree of Infection	No. of Colonies Exam.	Agglutination Reactions (Dilutions of 1:50 and 1:100)*	Type
122	38	2 mos. 14 days	±	2	Poly., ++; normal, ++; para, 0	Normal
123	38	2 mos. 14 days	+	1	Poly., 0; normal, ++; para, 0	Normal
135	32	1 mo.	±	1	Poly., 0; normal, ++; para, 0	Normal .
145	30	1 yr. 11 mos.	±	1	Poly., ++; normal, ++; para, 0	Normal
151	16	4 mos. 20 days	+	3	Poly., ++; normal, ++; para, 0	Normal

TABLE 5.

Group of Cultures Showing Sedimentation and Slight Agglutination with Flexner's Serum.

Serial No.	Age (Years)	Time in Jail	No. of Colonies Examined	Degree of Infection
41	25	24 days	1	±
106	29	1 mo. 28 days	1	±
114	45	1 mo. 1 day	1	±
131	39	6 days	1	±

The age of the inmate apparently had no bearing on the carriage of the meningococcus.

The length of time of detention in the jail previous to the taking of the culture is summarized in Table 6.

TABLE 6.

Time in Jail Previous to Making Cultures.

Number of	Up to 1 Month	1 to 3 Months	3 to 6 Months	6 to 12 Months	After 1 Year
Negatives:	32	31	10	13	12
Positives:	9	18	3	0	2

It will be noted from Table 6 that most of the inmates showing positive cultures had been confined up to three months. The epidemic was four to five months in its course when the cultures were taken. The inmate who carried the para type—the type prevailing in the epidemic—was confined twenty-nine days. Two others, infected with irregular para types—also found in a very few patients—were confined eleven and thirty-six days previous to the culture. It appears, then, that the older prisoners, under the conditions of life in this prison, apparently become to a great extent free from the meningococcus. It also appears that the few inmates who harbor the type prevailing in the epidemic have been recently confined.

With reference to the degree of infection, it is interesting to note that most of these carriers harbored only a few or occasional organisms. In 4 of the 32 carriers, however, they were numerous (++), and in two the meningococcus was the predominating organism (+++). The only inmate who carried a para type—the prevailing type of the epidemic—was lightly infected.

In several instances, more than one colony of the same plate culture was examined for type. The results confirm the already established fact that the meningococci isolated from the same nasopharynx are usually of the same type.

Finally, an analysis of the types found shows that only one inmate yielded a para organism. On the other hand, 34.3 per cent. of the carriers yielded the normal type and the remainder (excluding 5 in whom no serologic tests could be made) or 50 per cent. of the carriers, harbored irregular or inagglutinable types.

In conclusion, the results of the swabbing of a number of inmates of the jail show that 24.6 per cent. were carriers of organisms indistinguishable from meningococci; that these carriers yielded types which, generally, were not found among the patients, namely, normal, irregular or inagglutinable organisms. Also, the percentage of carriers in the jail was higher than that demonstrated by others among the contacts with patients during the epidemic. Finally, among the 600 or more prisoners, no case of epidemic meningitis developed.

Other Epidemiologic Factors.

In view of the fact that the following observations were made during an epidemic in a civil population, they are of interest for comparison with the experiences obtained in the Army and Navy.

(*a*). *The Influence of Age and Sex.*—From the local hospital records of 417 patients it will be noted that the ages of the patients ranged from 4 months to 59 years; the average age was 22.68 years. From the record of the Medical Officer of Health, based on the first 750 cases, mainly fatal, the great peak of the curve of age incidence is from infancy to 5 years; a lesser curve is seen at 17½ years.

The number of male cases at the local hospital was over twice that of the females; the official records show males to have been only slightly in excess of females. These figures when compared with the numbers of the sexes of the normal population, would be corrected so that the number of male cases is in excess of female only among the young adults.

TABLE 7.

Fluctuation of Number of Cases with Temperature Variations.

Date	Degree of Drop (Temp. of Saturation)	No. of Cases (Increase Over Previous Low Level)	Time Elapsed Between Drop of Temp. and Increase of Cases
2/14–2/18	About 13°C.	11	4 days
2/27–3/2	About 11°C.	8	4 days
3/10–3/12	About 9°C.	4	2 days
3/25–3/27	About 12°C.	15	5 days
4/9–4/11	About 14°C.	5	5 days

Therefore, the susceptible elements of the population are the children, with no definite sex preponderance, and young adult males.

(*b*). *Influence of Meteorologic Conditions.*—The temperature, the mean as well as the wet-bulb (temperature of saturation), had a marked influence on the incidence of the disease. In general terms it may be stated that when there occurred a sudden drop of temperature, the number of cases increased, and when there was a rise in temperature, the incidence of epidemic meningitis declined. From a chart prepared by the Medical Officer of Health, one may formulate a table to show this relationship (Table 7).

On the other hand, the effect of a rise of temperature on the incidence of the disease can be formulated as in Table 8.

Subsequently, as the temperature rose there was a corresponding decrease in the number of cases.

From the above it will be seen that as a rule about four days after a drop in temperature there was a great increase in the number of cases reported. The correspondence of this number with the number of days of the incubation period of epidemic meningitis is quite suggestive.

The influence of sunshine has also a relationship to the number of cases. For example, the period from February 28 to March 3 (four days) showed only 5½ hours of sunshine. Three days later, 23 cases were reported, the greatest number reported for a day with one exception. There was practically no sunshine for four days from March 26; on the fourth day after this period, 21 cases were reported, although there were only from 5 to 15 cases for the previous week. Thus, the lack of sunlight shows a relationship to the increase in the number of cases.

TABLE 8.

Effect of Rise of Temperature on the Incidence of the Disease.

Date	Degree of Rise (Temp. of Saturation)	No. of Cases (Decrease Over Previous Low Level for Week)	Time Elapsed Between Rise in Temp. and Decrease of Cases
2/18–2/27	About 20° C. (gradual rise)	8	8 days
3/15–3/24	About 17° C. (gradual rise)	4	8 days
3/31–4/9	About 13° C. (gradual rise)	4	4 days

It is doubtful whether humidity or rainfall by themselves have any bearing on the number of cases. January and February were extremely dry months. Indeed, the rainfall was considerably below the average for several years. Yet February saw increasing numbers of cases, and March with a relatively small amount of rainfall, the greatest number. Similarly with the humidity; increasing humidity bore no relation to the number of patients.

(*c*). *Pre-Epidemic Infections*.—It is a matter of common knowledge among the practitioners, although no official records are available, that many cases of sore throat were prevalent in the district during

the period preceding the epidemic (November and December, 1917). The ailment was not severe, but at times it resembled influenza. Its distribution, however, was widespread.

On the other hand, there is no evidence of any increase in the incidence of measles or mumps.

(*d*). *Migration of the Population.*—The effect of emigration[2] from the district on the widespread dissemination of the disease is clearly shown. A few weeks after the outbreak cases were noted (and in most instances for the first time) in five cities along the trade routes north and south of the district. The cases numbered from a few to sixty, and in one instance nineteen out of twenty cases yielded a parameningococcus, the prevailing type.

The influence of immigration, on the other hand, on the continuation of an epidemic suggests itself. The entrance of a new, susceptible element of population from a noninfected district and its existence in close contact with the disease should increase the number of cases. Or a disturbance of the ratio of insusceptibles of the native population is created, offering new soil for an outbreak. The extent of immigration may be inferred from the records of 1916 (72,405) and 1917 (98,232).

(*e*). *The Habits of the Natives.*—Certain infringements of the rules of hygiene which may have a special relationship to the spread of the disease are continually practiced by the Chinese of this district. The most flagrant are in connection with the street restaurants. The edibles are exposed to the open air and served in dishes which are not cleansed between the servings, so that what is in one man's mouth may be directly conveyed to another's. The other infringements relate to the use of common towels and drinking cups; the drying and sorting of food on the highways which are covered with expectoration.

(*f*). *The Overcrowding of the Population.*—As the epidemic was limited to a great degree to the overcrowded sections, it is important to describe this condition in detail.

Three causes operate to effect overcrowding. In the first place, there are too many houses on too small a space. Structures are in

2. The activity of emigration may be judged from the records for 1916 and 1917, namely, 117,653 and 96,298, respectively.

close proximity, separated from one another by narrow, chimney-like areaways. The lanes and areaways are so narrow, especially in proportion to the height of the houses, that not only ventilation but also light becomes deficient. The air is likewise damp almost all the time, from the wetness of the passageways.

In the second place, there are no arrangements for ventilation within the houses. For example, a house having a content of 5,818 cubic feet, has a window area of 51.8 square feet. One of the windows opens into a narrow street, the other into an oblong areaway of the diameter of a large steamship funnel. Besides, to avoid thieving or intrusion, the windows and doors are usually bolted, making the air very foul.

In the third place, there are too many inmates within a house. A floor of a dwelling is divided into boxlike compartments, called cubicles. There are from four to six cubicles to a floor. They are solidly partitioned off and the entrance is curtained, so that no air whatever is admitted. This is the case as a rule, although exceptions may be found in the first or last cubicle. A typical cubicle on investigation revealed that its content is 336 cubic feet of unventilated air space. This cubicle is the dwelling of six persons. It is a frequent occurrence to find twenty persons existing on a floor space of 270 square feet.

The tendency of the Chinese to overcrowd is noted throughout the community. The market is crowded, so are the streets, the street cars, the lodging houses, native schools and matsheds.

Hence, there are too many houses on too small an area; there are no sanitary arrangements for light and air within the houses; there are too many persons living within the houses, and there is an innate gregariousness all causing dense overcrowding.

(*g*). *Geographical Distribution of Cases.* —It is of interest to note that while it was of rare occurrence that more than one member of a family was afflicted, yet the greatest number of cases occurred in limited areas. Briefly, these areas comprised the most overcrowded sections. Indeed, in proportion to the same number of inhabitants, sparsely settled or less crowded areas yielded none, or an occasional case. Within the crowded district there are sections still more densely congested; the percentage of incidence to the population in the latter areas was three times that of the former.

Epidemiologic Conclusions and Discussion.

The conditions prevailing in the district have been stated, and an attempt will be made to draw from them epidemiologic conclusions. The conditions usually prevailing during a pre-epidemic period occurred here as well, namely, an extremely cold winter combined with a large number of cases of pharyngitis, bronchial affections, and possibly influenza. In view of previous experience, these circumstances are favorable for the development of cases of epidemic meningitis, but they do not explain the great spread of the disease in the district, especially in certain sections.

(*a*). *The Influence of Overcrowding.*—The factor of greatest importance in the dissemination of the disease is the extensive overcrowding. The bases on which this conclusion rests are:

1. The meteorologic conditions.
2. The relative incidence of the disease in crowded and in uncrowded sections.
3. The bacteriologic evidence obtained from the patients.
4. The relation of the carrier.

It has been demonstrated above that *meteorologic factors* have an important relationship to the dissemination of the disease; cold weather, especially in the absence of sunshine, resulted in an increase in the number of cases.

The effect of cold weather on the population is remarkable. As soon as it is felt, the natives abandon the habit of sleeping out in the open streets and highways, and literally swarm in the cubicles. These cubicles, as already stated, are boxlike compartments completely closed in and absolutely unventilated.

The mechanism of dissemination of the organism is more or less direct. It "consists in the ejection of the nasopharyngeal secretions into the surrounding atmosphere. This ejection does not take place during ordinary breathing, and little, as a rule, during quiet speaking. But in loud speaking and particularly in coughing, sneezing, hawking, and spitting, the secretions may be sprayed and scattered widely."[3] Should one inmate harbor the meningococcus, it will be soon dis-

3. Flexner, Simon: Mode of Infection, Means of Prevention and Specific Treatment of Epidemic Meningitis, J. A. M. A., 69: 639, 1917.

tributed over all the others of the dwelling. Thus, while the meningococcus is a very fragile organism and succumbs easily in external nature, in this condition of overcrowding and close contact induced by the cold weather, a general distribution is favored.

It is logical to assume that in a dark and damp atmosphere the viability of the organism is prolonged, favoring as well its spread from one to another.

The relative incidence of the disease in crowded and uncrowded sections adds further evidence. As shown in the foregoing, the disease took its greatest toll from the crowded districts. Among the foreign element an occasional case developed; among the Chinese it was common. Yet epidemic meningitis is not a disease peculiar to the Chinese.

On the other hand, in a neighboring city where there are four times as many Chinese, but not so densely settled, and where there is no cubicle system of dwelling, but the people live in ventilated rooms, not a single case developed among the natives.

The *bacteriologic evidence* obtained from patients is suggestive of the spread of the disease by close contact. Almost all (95 per cent.) the patients examined were infected with one type, the parameningococcus. As other types were found in the community, it is reasonable to assume (among other things) the rapid communicability of this type one to another—a result of the great density of the population.

The *relation of the carrier* in comparison with overcrowding was studied in the inmates of the jail. The results show that 24.6 per cent. of the 130 inmates examined were carriers of various types of meningococci. Yet at this institution not a single case developed. The important fact is that there are isolated cells there and no crowding.

Also among contacts, Europeans showed 9.8 per cent., while Chinese showed 6.7 per cent. of carriers. Yet the Chinese by far outnumbered the European cases.

This evidence leads to the conclusion that overcrowding is the important factor in the spread of the epidemic. At the same time, one must regard the carrier as a *sine qua non* as regards the source and the agent of transmission of the disease.

It appears, however, that the actual numbers of carriers present have only little epidemiologic significance. It is the type which is

carried that is significant. Patients yield para types; the prisoners, although within the epidemic area but among whom no case develops, yield practically only normal, irregular, and inagglutinable types. The question of their relationship is still under investigation. However, it appears to favor the view recently stated by Flexner[4] that in healthy carriers one type (the saprophytic), while in the epidemic cases another (the pathogenic), prevails.

(*b*). *Other Factors Contributing to the Continuance of the Epidemic.*—The epidemic having gained headway, it is possible that its continuance depended on the constant migration and the unhygienic habits of the natives; the many ways prevailing in the Chinese community of distributing nasopharyngeal secretions from one to another.

Recommendations for Prevention.

The local conditions led to a formulation of the following means of prevention. The efficacy of these means cannot be judged until the next cold season will have passed. In outline form they are:

(*a*). The education of the Chinese in order to effect an active cooperation with the health and sanitary authorities.

(*b*). The prevention of overcrowding.

(*c*). The prevention of droplet infection by the instruction of the principles of personal hygiene and the employment of masks.

(*d*). The detection and treatment of contact carriers including the isolation of the "dangerous" carrier (one who harbors numerous meningococci, particularly of the same type as the patient).

(*e*). The isolation of the patient.

(*f*). Although still in an experimental stage, the use of preventive inoculation of an antimeningococcic vaccine.

CONCLUSIONS.

In four of ten moribund cases of epidemic meningitis, the meningococcus was found circulating in the blood. It is difficult to interpret these findings with regard to the theory that blood invasion is primary or secondary, as in these cases the results were obtained antemortem.

4. Flexner, Simon: Control of Meningitis, J. A. M. A., 71: 638, 1918.

However, it emphasizes the need of intravenous combined with the intraspinous methods of treatment.

Serums having a low agglutinin content were therapeutically ineffective.

About 95 per cent. of the patients were infected with one type, the parameningococcus; the remainder with the irregular para type.

In a series of prisoners, who lived under hygienic conditions, 24.6 per cent. were found to be carriers. Of these, 50 per cent. yielded irregular or inagglutinable types and 34.3 per cent. the normal type, and only one person the para type. Although the jail is within the epidemic area, not a single case of meningitis developed therein.

These and other facts already stated have led to the conclusion that dense overcrowding of population and the presence of a pathogenic type of meningococcus, rather than the actual numbers of healthy carriers of various types of the organism, are the causes of the great spread of this epidemic.

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RELATION BETWEEN THYROID GLAND, METAMORPHOSIS, AND GROWTH.

By EDUARD UHLENHUTH.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, February 1, 1919.)

It has been demonstrated by a number of workers that the active principle of the thyroid gland in the causation of amphibian metamorphosis is iodine. Recently Swingle[1] has obtained definite proof that inorganic iodine when fed to larvæ of *Salientia* induces metamorphosis in a short time after the beginning of the feeding. On the other hand, it has been shown by the writer[2] that the retarding influence of the feeding of thymus upon amphibian metamorphosis is due to the absence from the thymus gland of a substance required for metamorphosis, and it is possible that this lacking substance is iodine. Although minute amounts of iodothyrin have been found in some thymus glands, it is likely that the amounts found are insufficient to produce metamorphosis and that some thymus glands may not contain the iodine at all. This would account for the variability of the results obtained with thymus feeding.

Swingle[1] also subjected tadpoles deprived of their thyroid to an iodine diet; although such larvæ when kept on a normal diet never metamorphose, they very soon metamorphosed when fed on iodine crystals.

Hence it is manifest that iodine is one of the substances involved in the causation of amphibian metamorphosis. The quantities contained in normal food are, however, so small as to have no immediate effect upon the organism. If the larvæ have no thyroid glands, the small quantities of iodine taken up with the food apparently cannot be retained by the organism and the iodine leaves the body without

[1] Swingle, W. W., *J. Exp. Zool.*, 1919, xxvii, 397.

[2] Uhlenhuth, E., *J. Gen. Physiol.*, 1918–19, i, 305.

bringing about metamorphosis. If, however, a thyroid gland is present, all or most iodine taken up by the organism is retained and stored up in the thyroid. At a certain time during the life of the larva the thyroid suddenly begins to excrete the iodine stored up during the larval period, and metamorphosis results.

From this it is evident that under normal conditions the iodine is not the only substance needed in metamorphosis; there must be still another substance which, when present in a certain quantity, causes the thyroid to excrete the thyroid hormone. The experiments to be reported in this article not only furnish evidence of the actual existence of such an excretor substance, but they also demonstrate that it is evolved during the processes which lead to the growth of the organism.

Metamorphosis and Rate of Growth in Worm-Fed Larvæ.

In eleven series of larvæ of the species *Ambystoma opacum* the rate of growth during the larval period of active growth has been determined in the following way.

In each series the time of metamorphosis was recorded for each single individual; this can be done very accurately in this species if the first shedding of the skin and the reduction of the gills to mere stumps without fringes are taken as the indication of metamorphosis, both phenomena occurring within a day. That these two processes are actually controlled by the influence of the thyroid, in contradistinction to many other processes, has been indicated already in former articles[3] and will be shown in detail in a later publication. From the values obtained in this way the average length of time of the larval period was calculated for each series (Table I).

Each individual was measured once a week and the average sizes obtained from those values for each series were plotted in curves. At the time of metamorphosis a sharp drop of the curve takes place, due to a discontinuation of growth, and even a diminution of the size of the animals which may last for one or several weeks before growth is resumed. In Series A 1916, the curve of which is shown in Fig. 1,

[3] Uhlenhuth, E.: *J. Exp. Zool.*, 1917-18, xxiv, 237; 1918, xxv, 135; *J. Gen. Physiol.*, 1918-19, i, 305.

this drop occurred in the 24th week, though less distinctly than in most of the other series. From the average sizes reached in each series when the drop occurred and from the time when the drop took place, the rate of growth was calculated in day-millimeters, as indicated in Column 3 of Table I.

In comparing the length of the larval period for each series with the corresponding rate of growth it becomes evident that the smaller the rate of growth observed, the later metamorphosis took place. Furthermore, the rates of growth appear to be proportional to the

TABLE I.

Rate of Growth (R) in Worm-Fed Ambystoma opacum during Larval Period of Active Growth (Calculated from Averages).

Series.	Age at metamorphosis.	R	R × A
	days.		
C 1916	245	0.27	66
A 1916	182	0.31	56
E 1917	161	0.36	58
D 1917	127	0.48	61
W_K 1917	106	0.58	62
W_{Na} 1917	100	0.58	58
W_{Mg} 1917	100	0.58	58
W 1917	97	0.62	60
W_{Ca} 1917	96	0.62	60
C 1917	80	0.79	63
XIV 1918	70	0.83	58
Average			60

velocity of metamorphosis; for the product of the rate of growth into the duration of the larval period (R × A) is constant, as may be seen from Table I, the average value of R × A being 60.

This means that during the process of growth a substance is evolved which when present in a definite amount induces metamorphosis, provided that the larvæ have been fed on normal food which apparently contains enough iodine to furnish the other substance (iodine) required for metamorphosis in a sufficient quantity; the greater the rate of growth the quicker that quantity of the first substance is formed which is required to induce the secretory action of

the thyroid gland. Hence besides iodine still another substance is needed in the amphibian metamorphosis; namely, the excretor substance which causes the thyroid to excrete the stored up iodine.

It may be mentioned here that the sudden drop of the growth curve at the time of metamorphosis may be explained if we assume that the thyroid, when it is stimulated by the excretor substance, excretes at

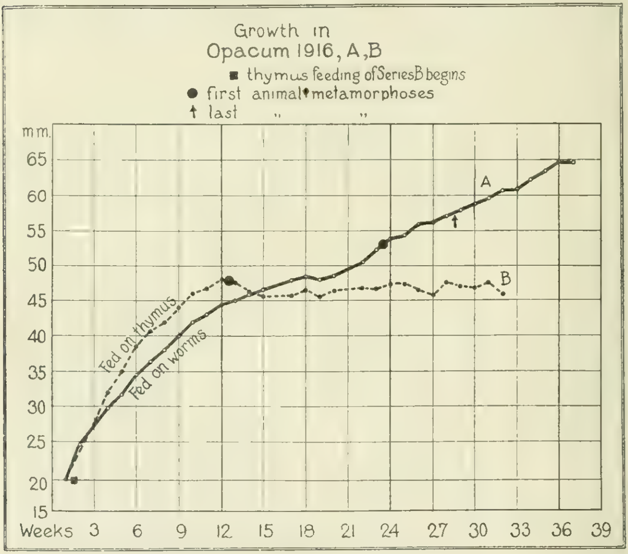

Fig. 1.

first an overdose of iodine, while later the excretion becomes less energetic. Janney[4] has shown that while an overdose of thyroid hormone leads to a negative nitrogen balance and a loss in weight and size, a certain minimum dose results in a positive nitrogen balance and a gain in weight and size.

[4] Janney, N. W., *Arch. Int. Med.*, 1918, xxii, 187.

Metamorphosis and Rate of Growth in Thymus-Fed Larvæ.

In a recent publication Janney[4] has shown that while a certain minimum amount of the thyroid hormone results in an increased protein breakdown, it finally leads to a positive nitrogen balance and an increase in weight and size, since it not only accelerates the protein breakdown but at the same time facilitates the assimilation of the nitrogen into the proteins of the body tissues. This is shown also in the metamorphosis of the amphibians where the initial breakdown of the tissues, as demonstrated, for instance, by Morse[5] on tadpoles, and the decrease in size, resulting probably, as suggested above, from the excretion of an overdose in the beginning of the functional period of the thyroid, are followed soon by an increase in size and weight.

On the other hand, Janney's experiments have shown that in certain diseases, such as exophthalmic goiter, the normal synthesis by the thyroid of the thyroid hormone from iodine and certain organic substances is disturbed, and on the basis of Swingle's experiments we may assume that in these diseases the thyroid is unable to retain the iodine consumed by the organism with the food. This assumption is supported by the fact, as mentioned by Janney, that in exophthalmic goiter the thyroid frequently is found very poor in iodine. But since in this case the excretory function of the thyroid remains undisturbed, the thyroid is excreting, instead of the hormone, certain substances, probably the indole-containing amino-acid tryptophane which normally is used to build up the hormone, but which in itself is toxic causing a permanent protein breakdown without facilitating assimilation of the food nitrogen; consequently a negative nitrogen balance is brought about and a permanent loss of weight.

If we apply this hypothesis to amphibian metamorphosis, we should expect that in such larvæ, which have not been able to take up an amount of iodine sufficient for metamorphosis and whose thyroid consequently was unable to develop the normal hormone, the action of the excretor substance would lead to a prolonged and increased loss in size of the larvæ, without resulting metamorphosis, since in these animals excretion by the thyroid would commence as soon as the excretor substance reached the amount required, but the excreted

[5] Morse, W., *Biol. Bull.*, 1918, xxxiv, 149.

substance would be the toxic substance tryptophane instead of the normal hormone.

This expectation is actually fulfilled in the thymus-fed larvæ of *Ambystoma opacum*, as is shown by Curve B, Fig. 1. In this curve is represented the growth of a series of larvæ of the same age and from the same mother as the larvæ used for the experiment plotted in Curve A; both series were kept under the same conditions, but while Series A was fed on earthworms, Series B was fed on thymus. The thymus-fed larvæ grew normally in the beginning—and in this particular series even more quickly than the controls, a fact explained by the writer in a previous paper.[6] At the 13th week we observe a sharp drop in Curve B and hereafter growth was stopped and never resumed again. Exactly the same results were obtained for the thymus-fed larvæ of *Ambystoma maculatum* and *Ambystoma tigrinum*.

The time when the drop of the curve takes place in the thymus-fed animals is that at which metamorphosis should have occurred if they had received normal food. This was not only suggested by the general appearance of these larvæ and by the fact that a few larvæ actually did metamorphose (in Series B 1916 only one larva metamorphosed at this time), but it can be proved if we calculate the time of metamorphosis from the value of the product R $\times$ A for the worm-fed larvæ. This has been done in Table II for eight thymus-fed series of *Ambystoma opacum*. Again the product R $\times$ A was calculated from the duration of the larval period and the rate of growth, the latter value being obtained as in the worm-fed larvæ from the growth curve up to the point where the drop occurred. First we notice that the product R $\times$ A (Table II) in the first three series is far above the average value of R $\times$ A as obtained for the worm-fed series. This is due to the fact that the larvæ of these series did not metamorphose at a time proportional to their rate of growth but much later, due to the absence of iodine at this time. The time at which they should have metamorphosed if iodine had been present in the food in a normal amount, can be calculated, however, from the product R $\times$ A in the worm-fed series, which is 60, and the rate of

[6] Uhlenhuth, E., *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 37.

growth in each particular thymus-fed series. We find, then, that in the first three series metamorphosis was greatly retarded (Column 5, Table II). In Series D 1916 metamorphosis should have taken place at 162 days instead of at 207 days, in Series B 1916 at 109 days instead of at 133 days, and in Series T_{Ca} 1917 at 113 days instead of at 122 days. In the other series the retardation of metamorphosis, if there was any at all, was only slight; how far this was due to the special treatment these thymus-fed animals received will not be discussed here. It may be mentioned, however, that in the last series (T 1917) the retardation of metamorphosis was prevented probably

TABLE II.

Rate of Growth (R) in Thymus-Fed Ambystoma opacum during Larval Period of Active Growth (Calculated from Averages).

Series.	Age at metamorphosis.	R	R × A	Age at which animal should metamorphose.
	days			*days*
D 1916	207	0.37	77	162
B 1916	133	0.55	73	109
T_{Ca} 1917	122	0.53	65	113
B 1917	122	0.50	61	120
T_{Na} 1917	122	0.50	61	120
T_{Mg} 1917	109	0.58	63	103
T_K 1917	104	0.57	59	105
T 1917	101	0.60	61	100
Average			65	

by adding parathyroid to the thymus diet; since the parathyroids are known to contain small amounts of iodine, it is probable that the thyroid of these larvæ was apparently able to store up enough iodine to permit normal thyroid excretion and metamorphosis when the excretor substance began to act.

The number of days at which the second thymus-fed series (B 1916) should have metamorphosed, *i.e.* at which the excretor substance began to act, is 109. This approaches very closely the time when the drop of the curve occurred (95 days), if we consider that the value 109 was calculated only from an average value of R × A.

If prevention of metamorphosis by the absence of iodine, but in the presence of a thyroid gland, is accompanied by a check in growth (for the reasons given above), we should expect that prevention of metamorphosis in the absence of a thyroid gland would not be accompanied by an abnormal check of growth. Hence amphibian larvæ deprived of their thyroids should be able to complete the normal growth of the species without any disturbances. That this is actually the case is shown in Allen's tadpoles which were deprived artificially of their thyroid glands and which reached frequently enormous sizes, becoming real giant larvæ. A similar phenomenon is found in such forms as *Typhlomolge rathbuni*, which for some reason do not develop a thyroid gland—as discovered by Emerson[7]—and hence remain permanently in a larval condition, but which seem able to grow for years without disturbances.

Thus the experiments reported in this article seem to prove that metamorphosis of the normal larvæ of *Ambystoma opacum* which possess a thyroid depends not only upon the presence of a sufficient amount of iodine in this gland but also on the presence of the action of a second substance inducing the excretion of the iodine by the thyroid gland. By means of this hypothesis we are able to explain why thymus-fed larvæ suddenly stop growing at the time when metamorphosis should occur, without, however, metamorphosing, and why species not possessing thyroid glands, such as the *Typhlomolge*, can complete their growth without disturbance.

Metamorphosis and Temperature.

There is still another phenomenon which lends itself readily to explanation on the basis of the assumption of an excretor substance. It is a well known fact that growth is retarded at low temperature, and since we have seen that the excretor substance is evolved during growth, it is not surprising that metamorphosis also should be retarded at low temperature. This has long been observed by many students of amphibian metamorphosis. But what remains unexplained is the fact that amphibian larvæ when kept in low temperature are always much larger at the time of metamorphosis than

[7] Emerson, E. T., *Proc. Boston Soc. Nat. Hist.*, 1905, xxxii, 43.

they would be if kept at high temperature. We have observed this phenomenon frequently and in looking for an explanation have tried to find a relation between the size of the larvæ and the age at the time of metamorphosis, similar to that existing between the latter quantity and the rate of growth. Such a relation, however, does not exist.

But if we assume the action of an excretor substance in metamorphosis, the phenomenon in question can be readily explained. Comparing R × A for the first worm-fed series (C 1916) in Table I, which was kept at a temperature of 10°C. below that of the other series, with the rest of the series of Table I, we observe that it is very high (10 per cent) above the average, which would indicate that metamorphosis in this series was more retarded than the corresponding rate of growth would demand. Since in this series a drop of the growth curve similar to the drop of the growth curve of the thymus-fed animals did not occur, this case of undue retardation of metamorphosis cannot be explained in the same way as in the thymus-fed larvæ, *i.e.* by asuming that from a lack of iodine in the thyroid gland a destructive compound was excreted by the thyroid. The only way to explain this case of retardation is to assume that at low temperature less excretor substance is evolved than at high temperature during an equal rate of growth processes. That this should be possible is not at all surprising, but was to be expected since it is well known that the temperature coefficients for different physiological processes may differ greatly. Loeb, for instance, pointed out that not only the temperature coefficients but also their variations at the lower and upper temperature scale differ considerably in different physiological processes.[8]

But if the amount of excretor substance produced by an equal rate of growth is less at low temperature than at high temperature, the animals kept at low temperature must grow longer at an equal rate than those kept at high temperature before that amount of excretor substance is produced which is required to bring about thyroid excretion. Consequently the low temperature larvæ must reach a larger size than the high temperature larvæ, before they can metamorphose.

[8] Loeb, J., Mechanistic conception of life, Chicago, 1912, 212.

The same phenomenon was observed also in the thymus-fed larvæ (Series D 1916, Table II) and since here the lack of iodine and that of the excretor substance are combined, it is not surprising that the value for $R \times A$ is still higher (28 per cent) above the average than in the low temperature series of the worm-fed larvæ (Series C 1916, Table I).

Thus with the hypothesis of an excretor substance we can explain a phenomenon which for a long time was confusing to experimental biologists as well as to systematists.

SUMMARY.

1. Two substances are involved in amphibian metamorphosis as studied in *Ambystoma opacum:* first, iodine, which is taken up by the food, and second, an excretor substance, which is evolved during the processes of growth and serves to induce the excretory function of the thyroid gland.

2. This explains why in larvæ, whose metamorphosis is inhibited by lack of iodine, growth is checked at the time when metamorphosis should occur; for at this time the excretor substance commences to act and this results, if iodine is absent, in the excretion by the thyroid of toxic substances which cause the breakdown of proteins and consequently a decrease in size of the larvæ.

3. Larvæ whose metamorphosis is inhibited by extirpation of the thyroid or by the hereditary lack of a thyroid (as is the case in *Typhlomolge*) can grow normally, since in them the action of the excretor substance cannot result in the excretion by the thyroid of a toxic growth-inhibiting substance.

4. At low temperature less excretor substance is produced than at high temperature during an equal rate of growth; therefore larvæ kept at low temperature reach a larger size than larvæ kept at high temperature, before they metamorphose.

The writer wishes to express his thanks to the Library of the Brooklyn Museum, and especially to Miss S. H. Hutchinson, for courtesies extended to him.

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A YEAST MEDIUM FOR PROLONGING THE VIABILITY OF THE MENINGOCOCCUS.

By FREDERICK EBERSON, Ph.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

At present the need is felt for a simple medium for prolonging the viability of the meningococcus. A variety of mediums have been devised with this object in view, but generally they are prepared with difficulty, as in the cases in which animal blood is used, or they do not sustain the organism a sufficient time. In this communication a medium is presented which is simple to prepare and prolongs the viability of the meningococcus for at least six weeks.

Yeast contains elements necessary for bacterial growth and maintenance. Ickert[1] found that certain organisms thrive on yeast peptone agar although free from meat infusion or meat extract. The following is an adaptation to the meningococcus of Ickert's findings, using a modified medium:

Preparation of the Medium.

Ten gm. of bakers' or brewers' yeast are macerated in 100 c.c. of water for twenty minutes, and the suspension is steamed for two hours, the temperature not exceeding 100 C. It is then clarified by adding Merck's dialyzed iron (5 per cent. ferric oxid) and filtering through glass wool. The first step in this clarification is not necessary unless a specially transparent medium is desired. To 1 per cent. agar containing 2 per cent. peptone and 0.4 per cent. potassium phosphate is added an equal amount of the prepared yeast. The reaction is adjusted to $p\text{H} = 7.6$, which, after heating, is reduced to 7.4. The medium is distributed, 10 c.c. in each tube, and is autoclaved for one-half hour at 15 pounds.

The result is a semisolid medium which is seeded by making stabs into it with a considerable amount of the inoculum. The tubes are sealed with paraffin or sealing wax and are kept at 37 C. until required.

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1. Ickert, F.: Presshefe und Hefeextrakt zur Nährbodenbereitung, Deutsch. med. Wchnschr. 44: 186, 1918; abstr. Daily Review of the Foreign Press, May 1, 1918, p. 147.

Peptone is not essential. Satisfactory results were obtained on a peptone-free medium.

Report of Experiments.

Several strains of meningococci, normal, irregular, and para, spinal and nasal, recent and old, were seeded in this medium, and at the end of six weeks were still viable. In several instances the organism was recovered after seven, and in another after eleven weeks. If, after initial incubation, the cultures are kept at room temperature (from 23 to 26 C.), the period of viability is three weeks. In peptone-free medium the organisms were still viable after one month.

To test for viability, a small amount of the medium is transferred to sheep serum agar slants. It is important to note that the meningococcus produces no visible change in the medium. Indeed, a dried substrate may yield actively growing organisms. Hence a test is a necessary procedure. At times the mere transplanting to sheep serum agar slants fails to yield a growth. In such cases a small amount (from 0.5 to 1 c.c.) of 1 per cent. glucose broth is added to the yeast medium, and after from twenty-four to forty-eight hours' incubation a transplant is made to the sheep serum agar slants.

Further work is necessary to determine whether this medium is suitable for repeated transfers. However, its advantage in keeping the meningococcus alive over a long period is obvious, especially in transporting cultures over great distances.

CONCLUSION.

Yeast-agar, a simple medium, easily prepared, prolongs the viability of the meningococcus for at least six weeks.

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THE SUSCEPTIBILITY OF NATURALLY NEPHROPATHIC ANIMALS TO ACUTE MERCURIC CHLORIDE INTOXICATION.*

By WILLIAM deB. MacNIDER.

(*The Laboratory of Pharmacology of the University of North Carolina, Chapel Hill.*)

(Received for publication, February 3, 1919.)

In a recent paper[1] which was concerned with a study of acute mercuric chloride intoxication in the dog, an analysis of the experiments permitted the classification of the animals into four groups. This grouping depended upon the severity of the intoxication as indicated by the final outcome of the poisoned animals, and upon the rapidity of the development and the severity of an acid intoxication which was shown by the animals in certain of the experiments. In this paper the observation was made of a relationship between the degree of acid intoxication and the time of appearance and the severity of the kidney injury.

In a more recent paper[2] which was concerned with a study of the acid-base equilibrium of the blood in naturally nephropathic animals and of the functional capacity of the kidney in such animals following an anesthetic, it has been shown that the acid-base equilibrium of the blood of such animals is not so stable as is the case for normal animals. When an anesthetic is given to such naturally nephropathic animals, there occurs a rapid depletion in the alkali reserve of the animal's blood, and associated with this change there develops a severe, acute injury to the renal epithelium. Such animals become acutely anuric and fail to respond to diuretic substances which in normal control animals are of distinct diuretic value.

The present investigation was undertaken with the object of ascertaining if the naturally nephropathic kidney gave evidence of showing any increased susceptibility to intoxications by mercuric

* Aided by a grant from The Rockefeller Institute for Medical Research.

chloride, and furthermore to determine if there existed any relation between the time of development and the severity of the kidney injury with the change which the mercuric chloride effects in the acid-base equilibrium of blood.

Fourteen naturally nephropathic animals have furnished the material for the experiments. At autopsy these animals have shown a type of chronic nephropathy previously described,[3] in which the chronic pathology is very largely a focal process confined to the glomeruli and consisting in both intracapillary and capsular changes.

The animals used in the experiments were kept in metabolism cages for two days prior to inducing the mercury intoxication. During this period, and following the use of the poison, the animals were catheterized twice a day, and the urine examined quantitatively for albumin by the use of both Esbach's and Tsuchiya's reagents. The percentage of albumin obtained by the latter reagent is always greater than when Esbach's reagent is employed. Centrifugalized specimens of urine were examined for casts. The functional capacity of the kidney was determined by the phenolsulphonephthalein test of Rowntree and Geraghty, and by noting the percentage retention of blood urea. Changes in the acid-base equilibrium of the blood were studied by determinations of the alkali reserve, and the tension of alveolar air carbon dioxide. The methods of Marriott were employed for these observations.

In the morning of the day of the experiment, the animals were given five hundred cubic centimeters of water by stomach tube. At 2 P.M. the animals were partially narcotized by a hypodermic injection of morphine sulphate. Half an hour later the animals were given fifteen milligrams of mercuric chloride per kilogram. The solution of mercuric chloride was of one per cent strength. The animals were returned to the metabolism cages and observations made every twelve hours as previously indicated.

The tables give in brief the findings in six naturally nephropathic animals before and after the intoxication by mercuric chloride. The number of the experiment used in the various tables refers to the same animal.

Reference to Table I. shows that all of the naturally nephropathic animals were freely diuretic on the day of the experiment. The

urine of all of the animals contained a trace of albumin and an occasional hyaline or granular cast. In all of the animals, with one exception, Experiment 5, the blood showed a retention of blood urea. The maximum retention was 0.021 per cent. Further evidence of the kidney injury was shown in the reduction of the elimination of phenolsulphonephthalein during a two-hour period. The maximum output of the dye, sixty-nine per cent, occurred in the animal of Experiment 5. In the remaining thirteen animals of the series, the elimination of the dye showed a greater reduction. The greatest reduction in elimination was forty-eight per cent in the animal of Experiment 1. A study in the changes of the acid-base equilibrium of the blood of the naturally nephropathic animals prior to the mercuric chloride intoxication shows that none of the animals had an acid intoxication. The reserve alkali determinations have varied between 8.0 and 8.1, while the tension of alveolar air carbon dioxide has not shown a reading below 38 mm.

A study of Tables II. and III. shows the effect of an acute mercuric chloride intoxication in the same animals which were used as representatives of the group in Table I.

All of the animals developed a gastroenteritis which varied in severity. The output of urine during the first twenty-four-hour period was reduced. The albumin of the urine, with two exceptions, was slightly increased. The amount of albumin was not over 1.5 grams per liter. The quantitative determination of the albumin content of the urine is no index of the severity of the kidney injury. During this period of the mercury intoxication, the elimination of phenolsulphonephthalein by the kidneys shows an abrupt reduction. In the animal of Experiment 6, the output of the dye was reduced to a trace which could not be determined. The maximum output was forty-one per cent in the animal of Experiment 2. With this indication of renal insufficiency, there occurs a retention of blood urea. This retention has been the greatest in those animals which have shown the greatest reduction in the elimination of phenolsulphonephthalein. Associated with the development of the acute kidney injury, there has occurred in all of the animals of the series a rapid reduction in the reserve alkali of the blood and a decrease in the tension of alveolar air carbon dioxide. The reduction in the tension

of alveolar air carbon dioxide is relatively proportionate to the change in the alkali reserve of the blood.

The general condition of the animals has shown a dependence upon the blood changes and the kidney injury. Two of the animals of the series at the end of this period of the intoxication were bright and were able to eat their food and retain it. As will be noted from Table II., these animals (Experiments 2 and 4) showed less evidence of an acid intoxication than the other animals, and had a phenolsulphonephthalein output of forty-one per cent and thirty-two per cent respectively. One of the animals (Experiment 3) died during the night. A second animal (Experiment 1) developed convulsions but lasted into the second day of the intoxication. The remaining animals were drowsy, with periods of restlessness.

A study of the course of the experiments during the second twenty-four-hour period of the intoxication, Table III., shows the output of urine by the various animals to be greatly reduced. The amount of albumin was small, not over 0.5 gram per liter. Two of the animals were anuric. At this stage of the intoxication the elimination of phenolsulphonephthalein was insufficient to permit a determination. The blood urea in all of the animals was increased. The highest retention was found in Experiment 6, in which the retention reached 0.177 per cent.

At this stage of the poisoning, all of the animals had a severe grade of acid intoxication. The maximum alkali reserve was 7.8. The minimum reading reached the very low figure of 7.65. The tension of alveolar air carbon dioxide varied between twenty-four to ten millimeters. All of the animals were semicomatose. Three of the number died in "air hunger," one died in a coma without the typical breathing of "air hunger," and one died in convulsions.

The histological study of the kidneys of these animals has shown the chronic glomerular pathology previously referred to. The acute renal injury which has been superimposed on the chronic pathology by the intoxication with mercuric chloride has consisted in an acute and severe swelling of the epithelium of the convoluted tubules, which is rapidly followed by necrosis. The tubules of the loops of Henle show a fatty infiltration. The vascular pathology consists in an acute engorgement of the glomerular vessels.

TABLE I.

Naturally Nephropathic Animals prior to Mercuric Chloride Intoxication.

Number of Exp.	Urine in 24 Hours.	Albumin.	Casts.	Blood Urea.	Phthalein.	R. p. H.	CO_2 Tension.
1	1,165 c.c.	Trace.	Few hyaline.	0.021%	48%	8.0	38 mm.
2	760 c.c.	1 gm.	Few hyaline.	0.018%	51%	8.05	40 mm.
3	815 c.c.	Trace.	Few hyaline.	0.019%	61%	8.0	42 mm.
4	665 c.c.	Trace.	Few hyaline and granular.	0.021%	64%	8.0	43 mm.
5	1,015 c.c.	Trace.	Few hyaline.	0.012%	69%	8.1	45 mm.
6	1,273 c.c.	Trace.	Few hyaline.	0.014%	57%	8.05	45 mm.

TABLE II.

Naturally Nephropathic Animals after Mercuric Chloride Intoxication. First Twenty-Four-Hour Period.

Number of Exp.	Urine.	Albumin.	Casts	Blood Urea.	Phthalein.	R. p. H.	CO_2 Tension.	Condition of Animal.	Termination.
1	150 c.c.	0.25 gm.	Few hyaline and granular.	0.027%	12%	7.8	28 mm.	Two convulsions.	
2	330 c.c.	1.5 gms.	Few hyaline.	0.020%	41%	7.95	38 mm.	Good.	
3	30 c.c.	1.2 gms.	Numerous granular.	0.038%	10%	7.85	31 mm.	Drowsy.	Died during night.
4	81 c.c.	Trace.	Numerous granular.	0.034%	32%	7.9	35 mm.	Good.	
5	142 c.c.	1.5 gms.	Numerous granular.	0.42%	21%	7.85	30 mm.	Drowsy, followed by restlessness.	
6	160 c.c.	Trace.	Few granular.	0.033%	Trace.	7.7	14 mm.	Restless. Labored breathing.	

TABLE III.

Naturally Nephropathic Animals after Mercuric Chloride Intoxication. Second Twenty-Four-Hour Period.

Number of Exp.	Urine.	Albumin.	Casts.	Blood Urea.	Phthalein.	R. p. H.	CO_2 Tension.	Condition of Animal.	Termination.
1	35 c.c.	0.5 gm.	Few hyaline and granular.	0.082%	0	7.7	21 mm.	Comatose.	Died in "air hunger."
2	0	No urine.	No urine.	0.097%	0	7.65	18 mm.	Comatose.	Died in "air hunger."
4	0	No urine.	No urine.	0.068%	0	7.8	24 mm.	Comatose.	Died in coma.
5	15 c.c.	Insufficient urine.	Numerous granular.	0.087%	0	7.7	12 mm.	Comatose.	Died in convulsions.
6	31 c.c.	Trace.	Few granular.	0.177%	0	7.7	10 mm.	Comatose. Muscular twitching.	Died in "air hunger."

From this summary of the results obtained in fourteen naturally nephropathic animals intoxicated by mercuric chloride, the following deductions are made:

1. The kidney of the naturally nephropathic animal is very susceptible to the toxic action of mercuric chloride.

2. The toxic effect of mercuric chloride, in so far as the kidney is concerned, has been associated with the development of an acid intoxication.

3. The severity of the acute changes occurring in the kidney have shown a correlation with the severity and duration of the acid intoxication.

REFERENCES.

1. MacNider, W. deB. A study of acute mercuric chloride intoxications in the dog with special reference to the kidney injury. Journ. Exp. Med., xxvii, 519, 1918.

2. MacNider, W. deB. The stability of the acid-base equilibrium of the blood in naturally nephropathic animals and the effect on renal function of changes in this equilibrium. I. A study of the acid-base equilibrium of the blood in naturally nephropathic animals and of the functional capacity of the kidney in such animals following an anesthetic. Journ. Exp. Med., xxviii, 501, 1918.

3. MacNider, W. deB. A pathological study of the naturally acquired chronic nephropathy of the dog. Part I. Journ. Med. Research, xxix, 177, 1916.

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YEAST AUTOLYSATE AS A CULTURE MEDIUM FOR BACTERIA.

BY I. J. KLIGLER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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The necessity for conserving meat and meat products during the war has rendered a search for cheaper sources of nutritive media for bacteria highly desirable. Douglas and Gordon in England, and more recently Meyer in this country, have proposed the use of peptic and tryptic digests of animal tissues as a substitute for meat extracts and peptones. These materials cheapen the cost of media but necessitate the use of a digestive enzyme, usually varying in potency, and of considerable amounts of valuable animal protein. It seemed that some non-animal protein might prove equally satisfactory.

Yeast naturally suggested itself as a possible substitute. Extracts of yeast have been used in the past to reinforce peptone culture media. Furthermore, yeast readily undergoes autolysis, in the process of which there are formed varying proportions of so-called peptones and amino acids, while the vitamines remain intact. Thus yeast autolysate may be expected to contain all the nitrogenous elements required for the growth of bacteria. However, the proportions of the individual nitrogenous elements vary with the conditions and duration of the autolysis. The extent of the influence which such variations may have on the growth of bacteria cannot be predicted on theoretical grounds. Hence it became necessary to search empirically for the conditions required to obtain from yeast a satisfactory medium for bacteria.

Method of Preparation.

In addition to its cheapness the yeast medium is, if anything, easier to prepare than meat extract or meat infusion media. Two hundred grams of drained or centrifuged brewer's yeast are suspended

Number of experiments	Composition of mixture	Initial P_H	Tests, number of	Time of incubation	P_H	Chemical tests: Nitrogen content per 100 cc. Total	Amino	P_2O_5 per 100 cc.	Ash per 100 cc.	Bacteriological: Broth Str.	Pneu.
				days		*grams*	*grams*	*grams*	*grams*		
1	200 cc. crude brewer's yeast to 1000 cc. distilled water, 30 cc. M/15 NaH_2PO_4, 10 cc. chloroform; incubated at 37°C.	5.0	1	2	6.1	0.0818	0.0429			+++	++
			2	4		0.1128	0.0617			++	+
			3	5	6.1	0.0991	0.0566			+++	+
2	Same mixture	6.0	1	2	6.5	0.0930	0.0492			+++	++
			2	4		0.1184	0.0662			+++	
			3	5	6.5	0.1103	0.0657			+	++
3	Same mixture	7.0	1	2	7.3	0.0846	0.0455			++	+++
			2	4		0.1243	0.0665			+++	+++
			3	5	7.6	0.1159	0.0640			+++	+++
4	400 cc. crude brewer's yeast 1000 cc. distilled water, 2 grams NaH_2PO_4, 10 cc. chloroform; incubated at 37°C.	6.1	1	4	6.1	0.348	0.1803			+++	+
5	Same mixture	6.5	1	4	6.5	0.379	0.2078			++	–
6	150 grams drained yeast, 1000 cc. distilled water, 2 grams NaH_2PO_4, 5 cc. chloroform; incubated at 37°C.	6.1	1	3	6.1	0.2520	0.1760	0.1176	0.384	++	+
7	200 grams drained yeast, rest same as no. 6	6.1	1	3	6.1	0.2892	0.1922	0.1688	0.485	+++	++
8	200 grams drained yeast, 1000 cc. distilled water, 2 grams NaH_2PO_4, 5 cc. chloroform	6.1	1	$2\frac{1}{2}$	6.1	0.2603	0.1314			+++	++
9	400 grams drained yeast, 1000 cc. distilled water, 2 grams NaH_2PO_4; adjusted to P_H and autoclaved	7.0	1			0.128	0.0340			–	–
10	Nos. 8 and 9 mixed before broth was prepared	10				0.2342	0.0898				
11	Broth, Fairchild peptone					0.3663	0.0522			+++	+++
12	Veal broth, Witte peptone (Meyer and Stickel)					0.3472	0.0359	0.2	0.4088		

Bacteriological tests					Toxin	
Broth						
ys. F.	Dys. Sh.	Diph.	Staph.	Indol B. coli	B. diphtheriae	B. dysenteriae
++	+++	+++	+++	+		
++	+++	+	+++	++		
++	+++	++	+++	++		
++	+++	+	++	+	Not tested	Not tested
++	+++	++	+++	+		
++	+++	++	+++	+		
++	++	++	+++	+++		
++	+++	++	+++	+++		
++	+++	++	+++	+++		
++	+++	++	+++	+++	Filtrate 10 day broth culture inoculated intraperitoneally into guinea pigs (1) 0.3 cc., no effect (2) 0.6 cc., died 3d day (3) 1.0 cc., died 2nd day	2 cc. filtrate 4 day broth culture in ear vein of rabbit; paralysis 48 hours. Died in seventy-two hours
++	+++	+	+++	+++	Same as above (1) 0.3—no effect (2) 0.6—no effect (3) 1.0—no effect	Same amount as above; no effect
++	+++	++	+++	+++	1.0 cc. filtrate of 10 day culture inoculated into each of 2 pigs. Recovered	
++	+++	++	+++	+++	Same as above 1 died in 2 days 2 died in 4 days	
++	+++	++	+++			Not tested
++	+++	−	+++			
++	+++	+++	+++	+++	+	+

in a liter of water, 2 grams of NaH_2PO_4 are added as buffer and the reaction adjusted by the addition of N/NaOH, to $P_H6.1$. Then 5 cc. chloroform are added and the mixture is thoroughly shaken and incubated at 37°C. for two days. It is necessary to shake the flask occasionally during the incubation, to maintain sterility.

At the end of the incubation period the reaction is brought to $P_H7.4$ and the autolysate heated in a water bath or in the Arnold for thirty minutes. It is then filtered through paper, tubed and autoclaved.

Agar is prepared by adding 15 grams of agar directly to 1 liter of the unfiltered autolysate, and stirring thoroughly to immerse and soften the agar shreds or powder. The mixture is then heated on the free flame or in the autoclave until the agar is completely dissolved. The reaction is then adjusted to $P_H7.4$, the mixture is heated on a water bath or in an Arnold for half an hour and the partially clear supernatant fluid is decanted to an Erlenmeyer flask or other vessel. The agar is then cooled and whole egg added to clear. The medium is finally steamed in the Arnold for one half to three quarters of an hour, filtered, tubed and autoclaved.

Special media may be prepared with yeast broth or yeast agar as a base according to the usual procedure.

Results.

Table I contains a summary of the experimental data on which this report is based. About 300 cc. of crude brewer's yeast or 200 grams of the drained material are sufficient to make 1 liter of broth, the total nitrogen and ash of which are approximately equal to, while the amino N is approximately four to five times as high as that of ordinary broth. The hydrogen concentration at which autolysis is allowed to proceed is an important factor in determining the end products and the suitability of the filtrate for culture media. The optimum concentration is $P_H6.1$.

Brewer's yeast was used in all the experiments reported in this paper. Both the crude material and the drained and centrifuged yeast were tested. Comparative tests were also made to determine the effect of heat or filtration on the efficacy of the medium. Chemical analyses of the total nitrogen and amino acid content of the

autolysate media were made to parallel the cultural tests. The presence of tryptophane was determined by the ability of *B. coli* to produce indol. Toxin production in the case of *B. dysenteriae* and *B. diphtheriae* was tested by animal inoculation.

As a result of these experiments it may be concluded, tentatively at least, that yeast autolysate furnishes a suitable medium for the cultivation of all the common bacteria. The various members of the colon typhoid group thrive better in this medium than in ordinary broth. *B. coli* produces an abundance of indol in twenty-four to forty-eight hours. The Shiga type of *B. dysenteriae* on incubation of three to four days at 37°C. yields a toxin which produces the typical reaction in rabbits—paralysis after forty-eight hours, with typical intestinal lesions. The streptococcus and staphylococcus grow readily, but the pneumococcus does not thrive on the yeast autolysate media. The *B. diphtheriae* grows as well on these media as on ordinary agar or in broth. In the yeast broth it produces toxin, but apparently not of as high a potency as in some peptone broths.

It is not possible at present to predict the extent of the usefulness of yeast autolysate in culture media. Preliminary tests indicate that it can be used as a base for the various special media. Endo and brilliant green media prepared with yeast agar give entirely satisfactory differentiation.

SUMMARY AND CONCLUSIONS.

Experiments are presented indicating that yeast permitted to undergo autolysis may serve as a cheap substitute for more expensive animal proteins or their digestion products. The autolysate contains a high percentage of amino nitrogen and a relatively small amount of the higher nitrogen complexes. The fact that some bacteria, notably pneumococcus, meningococcus, etc., do not thrive as well in the yeast broth as they do in beef infusion media, would indicate that the higher nitrogen complexes,—polypeptides, etc.—play some part in bacterial nutrition. On the whole, however, it seems that the yeast autolysate media are entirely satisfactory for the cultivation of the less delicate pathogenic and saprophytic bacteria. Endo and brilliant green plates made with this medium give entirely satisfactory results.

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THE EFFECT OF CARBON DIOXIDE IN THE CULTIVATION OF THE MENINGOCOCCUS.

By FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 15 and 16.

(Received for publication, January 29, 1919.)

The experiments here reported are the outcome of observations made in applying the recommendation of Cohen and his associates[1,2,3] that meningococci be grown at a partial oxygen tension, obtained by substituting carbon dioxide for approximately 10 per cent of the air in a closed container.

The moisture requirement of the meningococcus has been repeatedly emphasized,[4] and it seemed important to determine whether Cohen's results might not be due, in part at least, to the retention of moisture in the partial tension chambers. In our series of carrier examinations two sets of carrier plates were incubated in moist chambers; one set in air, the other in the presence of carbon dioxide, from 2 to 25 per cent by volume. Our uniform experience has been that nasopharyngeal strains of meningococci grow as well, or better, in air saturated with water vapor in a closed chamber as in a similarly saturated atmosphere containing a small percentage of carbon dioxide. The conditions governing the luxuriance of the growth are factors of the humidity of the chamber and the qualities of the medium, and not of the slight differences in oxygen tension resulting from the replacement of a small part of the air with carbon dioxide.

Recently the opportunity presented itself at Camp Zachary Taylor of making similar comparisons with growths of meningococci

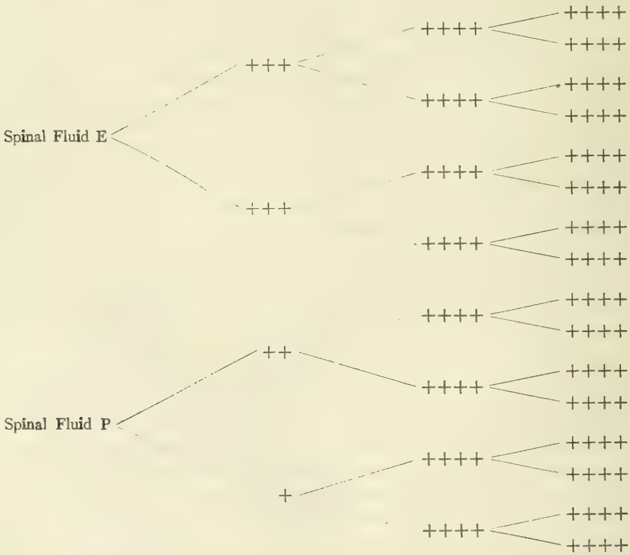
[1] Cohen, M. B., and Markle, L., A method which greatly facilitates the culture of the meningococcus, *J. Am. Med. Assn.*, 1916, lxvii, 1302.

[2] Cohen, M. B., Cultivation of the meningococcus under partial oxygen tension, *J. Am. Med. Assn.*, 1918, lxx, 1999.

[3] Cohen, M. B., and Fleming, J. S., The diagnosis of epidemic meningitis and the control of its treatment by rapid bacteriologic and serologic methods, *J. Infect. Dis.*, 1918, xxiii, 337.

[4] Lloyd, D. J., On vitamines, amino-acids, and other chemical factors involved in the growth of the meningococcus, *J. Path. and Bacteriol.*, 1916-17, xxi, 113.

from spinal fluids. As a routine approximately 2 cc. of spinal fluid were added to each of six slants of medium.[5] Three tubes of each set were incubated in a water-sealed chamber, in air, while three were incubated in a similar chamber containing 10 per cent carbon dioxide. No significant difference could be observed in the growths under these conditions. The slight differences noted favored the chamber containing air alone. Successive generations were also grown alternately in air and in 10 per cent carbon dioxide, without visible diminution in luxuriance. Two typical strains may be charted by the scheme by which Cohen has illustrated his results. Upstrokes indicate cultures in air, downstrokes cultures in 10 per cent carbon dioxide. One plus sign indicates a single colony, two indicate several colonies, three many, and four a confluent growth.



[5] Veal infusion agar, with glucose 1 per cent, adjusted to pH 7.4, to which 5 per cent sterile, unheated, citrated horse plasma was added just before tubing. This is a medium highly favorable to the meningococcus.

From these and similar findings the conclusion was reached that the replacement of 10 per cent of air by carbon dioxide has no appreciable effect on the growth of either nasopharyngeal or spinal strains of meningococci, under the conditions of our experiments.

Cohen considers that the effect of carbon dioxide, as he describes it, is not specific for this gas: that it acts merely as a neutral agent, displacing oxygen, and he calls the meningococcus a "microaerophile." A brief consideration of the composition of air, and of the actual displacement of oxygen by 10 per cent carbon dioxide shows that only approximately 2 per cent of oxygen would be displaced and its concentration reduced from 20 per cent to 18 per cent. Such a slight diminution could hardly be expected to produce the marked effects that Cohen reports. Moreover, if the action of carbon dioxide were not specific, results similar to Cohen's should be obtained with any neutral gas, such as nitrogen, which is chemically so inert. The fact that air is composed largely of nitrogen, and that only peculiar species of bacteria are able to utilize nitrogen from the air would seem to make it the most desirable agent for replacing oxygen in testing various oxygen tensions. Therefore nitrogen was used to replace oxygen in the following experiment.

Experiment 1.—January 13, 1919.

Eight plates of veal infusion agar, pH 7.4, with 5 per cent fresh sterile rabbit serum were inoculated with a suspension of a recently isolated spinal strain of normal meningococcus. Two of these plates were placed in each of four large Novy jars containing a little water. In three of the jars concentrations of nitrogen of 10, 25, and 50 per cent, in addition to that already present in the air, were obtained by partial exhaustion of the air and replacement with the gas from a tank. Thus partial oxygen tensions of approximately 18, 15, and 10 per cent were produced in the jars. The fourth jar served as an air control.

After 16 hours incubation the growths of meningococcus were profuse and practically identical in air, in 18 per cent oxygen, and in 15 per cent oxygen. Luxuriant confluent streaks of organisms showed the path of the inoculation. On one of the plates in 10 per cent oxygen the growth was poor, in the other very poor. Only a few small confluent masses of growth showed the site of heaviest inoculation. A plate from each jar is reproduced in Plate 15, Petri Plates 5 to 8.

The meningococcus did not thrive on artificial medium when deprived of half its accustomed oxygen tension. But this experiment does

not indicate that variations of 2 to 5 per cent below normal make any essential difference in its growth. What effect would increased oxygen tension have?

Experiment 2.—January 14, 1919.

Plates similar to those used in Experiment 1 and inoculated as before were divided among the four Novy jars. In a similar manner oxygen was used to replace the exhausted air in percentages of 5, 15, and 25, making the oxygen tensions of the atmospheres in the jars approximately 24, 32, and 40 per cent. The fourth jar again contained air alone.

After 16 hours incubation the growths in all the jars were profuse. Beside the confluent growths where the plates were most heavily inoculated large single colonies of luxuriantly growing organisms appeared. The meningococcus grew as well in 40 per cent oxygen as in air (Plate 15, Petri Plates 1 to 4).

Provided the oxygen tension is sufficient to support its growth, the meningococcus does not seem to be exacting about the concentration of its oxygen supply. These experiments show that the effects of increased carbon dioxide tension, as described by Cohen, are not due to the displacement of oxygen and that the meningococcus is not a "microaerophile." The action of carbon dioxide must be inherent in some specific property of the gas. Carbon dioxide in solution acts as a weak acid and might have a chemical effect on the medium in which meningococci are grown and on the organisms themselves. If serum agar plates, such as are used for isolating the meningococcus, are incubated in a moist atmosphere containing 10 to 30 per cent carbon dioxide, their reaction may be shifted toward the acid end of the pH scale. The following experiment shows this reaction and its effect upon a recently isolated (fourth generation) spinal strain of a normal meningococcus.

Experiment 3.—January 3, 1919.

Four 100 cc. lots of veal infusion agar were adjusted to the following reactions.

	Titratable acidity (phenolphthalein). *per cent*	pH concentration (phenolsulfonephthalein).
Lot 1	1.6	7.2
" 2	0.8	7.8
" 3	0.4	7.9
" 4	0.3	8.0+

To each lot were added 5 cc. of sterile, unheated rabbit serum, and eight plates were poured. Four plates of each set were heavily inoculated with a

suspension of meningococci from a third generation slant of a spinal strain. Eight plates (one inoculated and one sterile from each lot) were placed in each of four large Novy jars. In three of the jars concentrations of carbon dioxide of 10, 20, and 30 per cent were obtained by partial exhaustion of the air and replacement with the gas from a tank. The fourth jar served as an air control. After 18 hours incubation at 37.5°C. the growths of meningococci were noted and the pH concentrations of the uninoculated plates read with phenolsulfonephthalein and cresol purple as the indicators.

TABLE I.

Set No.	Original titer.		Final titer (pH).				Growths.			
	Acidity.	pH	Air.	Carbon dioxide.			Air.	Carbon dioxide.		
				10 per cent.	20 per cent.	30 per cent.		10 per cent.	20 per cent.	30 per cent.
	per cent									
1	1.6	7.2	7.4	7.0	6.7	6.4	Profuse.	Profuse.	Profuse.	Fair.
2	0.8	7.8	8.0	7.4	6.8	6.6	Good.	"	"	"
3	0.4	7.9	8+	7.4	6.8	6.6	None.	"	"	Good.
4	0.3	8+	8+	7.6	6.8	6.7	"	"	"	Profuse.

Table I shows the results, and Plate 16 the growths obtained in air, in 10 per cent carbon dioxide, and in 30 per cent carbon dioxide. The growths in 10 and 20 per cent carbon dioxide were practically identical. Several points are to be noted in an analysis of these findings.

The meningococcus does not grow in a medium of a titratable acidity of +0.4 or less when this corresponds to a pH value greater than 7.8 to 8.0 on the Sörensen scale. It may grow luxuriantly on a medium with a titratable acidity of +1.6 when this corresponds to pH 7.2. The fallibility of the old method of titrating media is clearly seen.

The partial saturation of a medium with carbon dioxide from an atmosphere containing 10 to 30 per cent of the gas may increase its hydrogen ion concentration markedly. Almost regardless of the original hydrogen ion concentration, the final reaction seems to represent a state of equilibrium between the carbon dioxide in the medium and in the surrounding air. Thus in the present experiment 10 per cent carbon dioxide brought the more alkaline plates to ap-

proximately pH 7.4 to 7.6, 20 per cent brought them to pH 6.8, and 30 per cent brought them to pH 6.6 to 6.7.

It is evident that such an action of carbon dioxide on the medium might exert either a favorable or an unfavorable effect upon meningococci, according to the original reaction of the medium. A favorable effect is seen in Petri Plates 3 and 4 of all the carbon dioxide series—a medium too alkaline to support growth was brought to a hydrogen ion concentration favorable to the growth of the meningococcus. An unfavorable effect, on the other hand, is seen in Petri Plates 1 and 2 of the carbon dioxide 30 per cent series; the medium was rendered too acid, and the meningococcus grew less well than in air.

Cohen's contention that meningococci which have developed aerobically do not grow well in the presence of carbon dioxide and *vice versa* is not borne out by this experiment, in which a strain isolated and grown for three generations aerobically grows well in carbon dioxide on a medium in which it cannot grow in air. In connection with the first experiment it may be pointed out that a meningococcus isolated and subcultured in air grew equally well under slightly reduced oxygen tension.

Cohen's reports indicate that this effect of carbon dioxide on the medium has operated favorably in his experiments, and thus the discrepancy between his results and ours may be explained. He and Fleming[3] describe the use of media 0.2 to 0.3 per cent acid to phenolphthalein, under the impression "that the meningococcus will not usually grow when the reaction is over plus 0.5 [per cent] acid to phenolphthalein." In serum agar, 0.2 to 0.3 per cent acid usually corresponds to pH 8.2 to 7.8 on the Sörensen scale, according to the amount of buffer present. Kligler[6] has recently found that the range of growth of the meningococcus in serum dextrose broth is pH 6.1 to 7.8, with the optimum at pH 7.4, and this fact, in conjunction with the effect of carbon dioxide in shifting the reaction toward the acid side, as shown above, suggests that incubation in a partial tension of carbon dioxide has made Cohen's media less unfavorable to the meningococcus, and so, combined with the undoubted aid of

[6] Kligler, I. J., personal communication.

moisture in the closed container, produced the results that he describes.

As for the effect of carbon dioxide on the organisms themselves, a specific action is not so easily demonstrated. That partial tensions up to 30 per cent may not be injurious is shown by the growths on the less acid plates of this series. On the other hand, Shaw-MacKenzie[7] states that carbon dioxide, 22 volumes per cent in Ringer-Locke solution, causes the death of the meningococcus in 20 minutes and suggests that "even the CO_2 normally occurring in the plasma and body fluids may form part of the protective processes of the body."

SUMMARY.

The meningococcus is not a "microaerophile." It grows equally well in atmospheres containing from 15 to 40 per cent oxygen.

If small amounts of carbon dioxide affect the growth of the meningococcus on an artificial medium it is by changing the reaction of the medium, not by slightly reducing the oxygen tension of the surrounding air.

The fallibility of titrating the total acidity of a medium is again clearly demonstrated. A reaction favorable to the meningococcus cannot be determined from the total titratable acidity but depends solely upon the hydrogen ion concentration of the medium. The optimum for the meningococcus is approximately at pH 7.4.

The value of a moist chamber in the cultivation of the meningococcus is shown by unusually luxuriant growth when other conditions are also favorable.

Addendum.

Since the completion of these experiments and the receipt of this paper for publication, St. John (St. John, J. H., *Med. Rec.*, 1919, xcv, 184) has reported his experiments on "Oxygen tension in its relation to the meningococcus." He concludes that the oxygen tension factor is at least of minor importance in comparison with the effect of moisture in promoting the growth of meningococci. He calls

[7] Shaw-MacKenzie, J. A., Toxic action of carbonic and other weak acids on the meningococcus, *J. Roy. Army Med. Corps*, 1918, xxxi, 1.

attention to the fact that the meningococcus is known to be especially sensitive to the degree of moisture and the reaction of the medium, and suggests that "the reaction of the meningococcus medium may be favorably influenced by certain gases evolved by the growing *B. subtilis*, but this consideration is also minimized by comparison with moisture controls."

At the time that St. John's article appeared there was in press a paper by Frederick L. Gates and Edgar T. H. Tsen on "The effect of moisture on the growth of the meningococcus" which was to have appeared in this number of *The Journal of Experimental Medicine*. Our findings on the importance of moisture in the cultivation of the meningococcus merely corroborated St. John's, and added nothing but emphasis to his conclusions. Our paper was therefore withdrawn from publication. We feel, however, that the degree of humidity most favorable to the meningococcus is not to be obtained easily unless closed containers are employed.

EXPLANATION OF PLATES.

Plate 15.

The effect of different oxygen tensions on the growth of the meningococcus.

Petri Plates 1 to 3. 16 hour growths of a spinal strain under increased oxygen tension. Petri Plate 4. A control growth in air.

Petri Plate 5. A control growth in air. Petri Plates 6 to 8. 16 hour growths of a spinal strain under decreased oxygen tension.

Plate 16.

The effect of carbon dioxide on the growth of the meningococcus, caused by changing the hydrogen ion concentration of the medium.

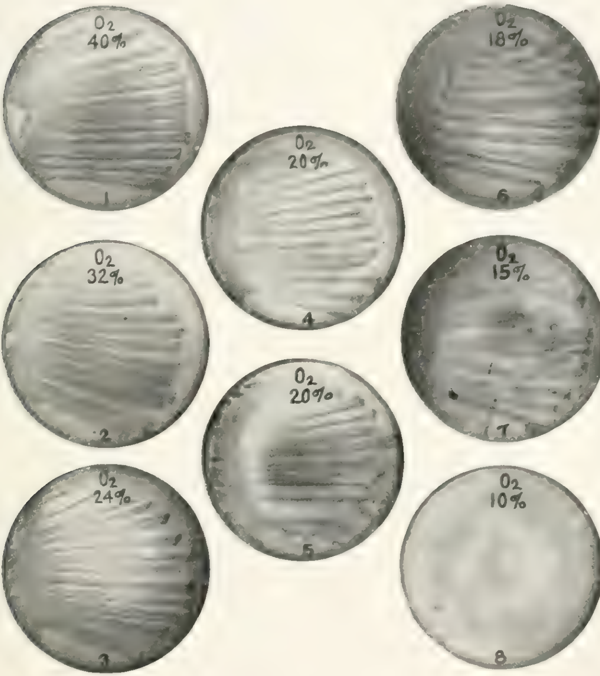
Petri Plates 1, 1, 1. Original pH 7.2; titratable acidity 1.6 per cent.

Petri Plates 2, 2, 2. Original pH 7.8; titratable acidity 0.8 per cent.

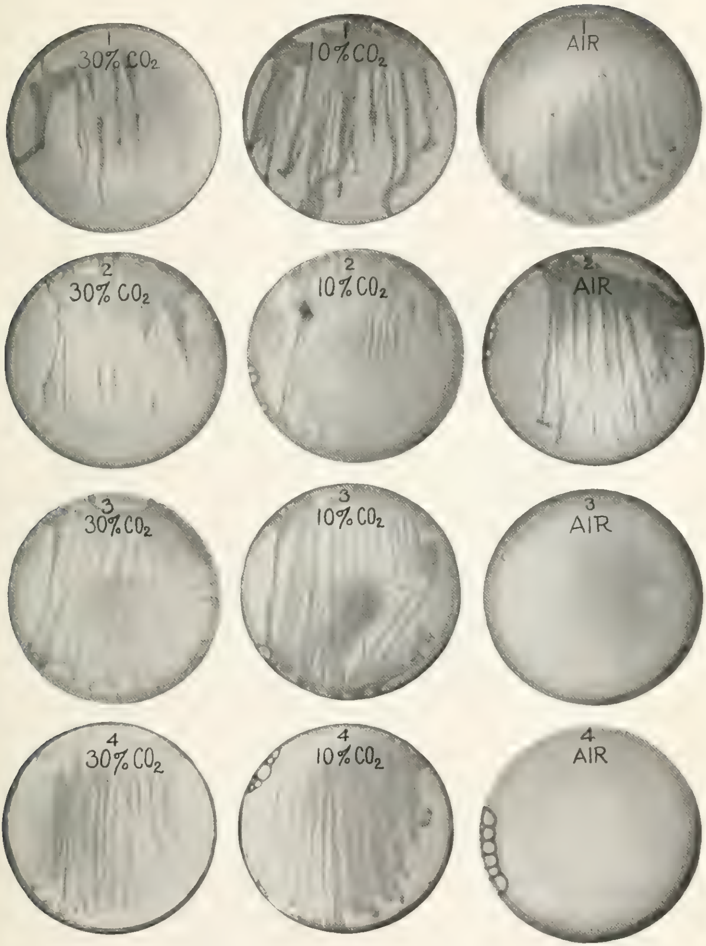
Petri Plates 3, 3, 3. Original pH 7.9; titratable acidity 0.4 per cent.

Petri Plates 4, 4, 4. Original pH 8+; titratable acidity 0.3 per cent.

In Sets 1 and 2, 30 per cent carbon dioxide increased the hydrogen ion concentration beyond the zone favorable to growth. In Sets 3 and 4, 10 and 30 per cent carbon dioxide brought plates originally too alkaline to support growth into the zone of hydrogen ion concentration favorable to the growth of the meningococcus.



O_2 40%
1
O_2 32%
2
O_2 24%
3
O_2 20%
4
O_2 20%
5
O_2 18%
6
O_2 15%
7
O_2 10%
8



(Gates: Cultivation of meningococcus.)

[Reprinted from The Journal of Experimental Medicine, April 1, 1919, Vol. xxix, No. 4, pp. 361-368.]

MIGRATION OF PARASITES AS THE CAUSE OF ANEMIA IN ÆSTIVO-AUTUMNAL MALARIAL INFECTIONS.*

By MARY R. LAWSON, M.D.

(*From the Laboratory of Dr. Mary R. Lawson, New London.*)

Plates 17 to 19.

(Received for publication, December 31, 1918.)

Malarial Anemia.

Malarial infections are characterized by the early development of a more or less severe anemia. As a rule, the æstivo-autumnal infections present the gravest manifestations. The severity of the anemia and its rapid appearance have frequently been noted.

Rossoni[1] states that no acute infection results in so active a deglobulization as does malarial fever, and that in all cases there is an immediate diminution in the number of corpuscles and the amount of hemoglobin. Manson[2] writes: "We find a degree of oligocythæmia greatly in excess of anything we might expect, or which can be accounted for by, or in correspondence with, the proportion of corpuscles attacked and directly consumed by the plasmodium."

Loss of Red Corpuscles in the Intervals between the Paroxysms.

Each parasite destroys several red corpuscles in the course of its development, the result of which is not only the loss of corpuscles with each paroxysm, but a loss in the intervals between paroxysms. That this interval loss has been noted might be understood from the following observations.

* Aided by a grant from The Rockefeller Institute for Medical Research.

[1] Rossoni, quoted from Thayer, W. S., and Hewetson, J., *Johns Hopkins Hosp. Rep.*, 1895, v, 60.

[2] Manson, P., Tropical diseases, a manual of the diseases of warm climates, London, 3rd edition, 1903, 88.

Thayer and Hewetson[3] state that in pernicious cases the number of red corpuscles may fall between the paroxysms. Marchiafava and Bignami[4] observed that anemia is produced in cases of pernicious fever which are not accompanied by elevations of temperature, the so called larval or masked pernicious fevers. They state: "Thus a patient of vigorous constitution in the first four days of a quotidian fever, or a remittent fever of first invasion, may show a reduction to 2,000,000 red blood corpuscles, or there may even be a reduction of 1,000,000 per cubic millimetre within twenty-four hours." Rossoni[5] states that the loss in hemoglobin and corpuscles is rarely evident during the paroxysm, but begins with apyrexia and may continue for several days afterwards. Dionisi[6] in a case of æstivo-autumnal infection observed a reduction of 500,000 red corpuscles in 12 hours, and he noted a fall from 3,200,000 to 2,300,000 corpuscles during the first 6 days of apyrexia in spite of the use of iron. Kelsch[7] saw a diminution of red corpuscles as low as 500,000 per c.mm. This diminution occurs, according to him, irregularly. Ewing[8] noted that the anemia may progress in the afebrile periods.

I have observed a continued loss of red corpuscles in the intervals between paroxysms.

Theories Advanced to Account for an Anemia Out of Proportion to the Number of Parasites Present.

The belief has been general that the only actual destruction of red corpuscles by parasites occurred when the parasites segmented, the destruction of the corpuscles corresponding to the segmentation of the parasites. Therefore when an anemia occurred out of proportion to the number of parasites present, provided each parasite destroyed but one corpuscle, certain theories were formulated to account for it.

[3] Thayer, W. S., and Hewetson, J., *Johns Hopkins Hosp. Rep.*, 1895, v, 57.

[4] Marchiafava, E., and Bignami, A., in Stedman, T. L., Twentieth century practice, New York, 1900, xix, 187, 188.

[5] Rossoni, quoted from Thayer, W. S., and Hewetson, J., *Johns Hopkins Hosp. Rep.*, 1895, v, 61.

[6] Dionisi, quoted from Ewing, J., Clinical pathology of the blood, New York, 2nd edition, 1903, 459, 460.

[7] Kelsch, quoted from Mannaberg, J., The malarial parasites. A description based upon observations made by the author and by other observers, translation by Felkin, R. W., London, 1894, 384.

[8] Ewing, J., *J. Exp. Med.*, 1900–01, v, 460.

Mannaberg[9] states: "It is possible that damage to the non-infested blood corpuscles is due to the parasitic poison which is dissolved in the *liquor sanguinis*. So we see that the red blood corpuscle in malarial patients may be attacked in two ways, but their destruction is far more due to direct invasion by the parasites than to the dissolved poison." Marchiafava and Bignami[10] write: "Nothing, however, forbids a belief that a special toxin of parasitic origin causes destruction of the red corpuscles by a specific action of its own. . . . . although the researches so far pursued with the purpose of directly proving the existence of poisons caused by the parasites have given negative results." Thayer[11] states "that there may well be other substances present in the circulation which result in the destruction of a number of non-parasitiferous elements." Ewing[12] writes: "Many factors indicate that the post critical anæmia is principally referable to globucidal action of the serum dependent upon the presence of a malarial toxin," one of the facts in support of this view being the "disproportion between the anæmia and the number of parasites present." Viola[13] believes that the destruction of red corpuscles depends only in a minor degree upon the direct action of the parasites and to a greater extent upon their toxic products.

As far as I can ascertain there has been no convincing evidence in support of the above views.

The Hemoglobin in Malarial Infections.

Reduction of hemoglobin out of proportion to the loss of red corpuscles is frequently observed. This reduction is easily explained. Two facts should make it clear: (1) a total destruction of red corpuscles accompanied by (2) a partial destruction by the parasites. There are at all times a certain number of parasites attached to corpuscles which are partially decolorized by parasitic action. When a red count and hemoglobin estimate are taken, each partially decolorized corpuscle counts as a corpuscle, and there is a partial loss of hemoglobin plus the total loss.

[9] Mannaberg, J., The malarial parasites. A description based upon observations made by the author and by other observers, translation by Felkin, R. W., London, 1894, 384.

[10] Marchiafava, E., and Bignami, A., in Stedman, T. L., Twentieth century practice, New York, 1900, xix, 199.

[11] Thayer, W. S., Lectures on the malarial fevers, New York, 1897, 185.

[12] Ewing, J., Clinical pathology of the blood, New York, 2nd edition, 1903, 461.

[13] Viola, quoted from Marchiafava, E., and Bignami, A., in Stedman, T. L., Twentieth century practice, New York, 1900, xix, 193.

Migration of Parasites in Æstivo-Autumnal Infections (Figs. 1 to 93).

It is practically impossible to ascertain the total number of migrations in the æstivo-autumnal infections as so few of the developmental stages are present in the blood. Migration of parasites is undoubtedly going on in the internal organs with consequent loss of red corpuscles. As stated above, each parasite destroys more than one red corpuscle; that is, after destroying one corpuscle, the parasite migrates to another and proceeds to destroy that. In the intervals between attachments the parasite is, for brief periods of time, free in the blood serum. All the parasites of one brood do not migrate at one time any more than do all the parasites of one brood segment at once.

In films containing free parasites in migration (Figs. 1 to 30, 35 to 64, and 66 to 69) one finds, usually, other parasites in similar stages of development, attached to dehemoglobinized red corpuscles (Figs. 65 and 86 to 93) or to corpuscular skeletons (Figs. 31 to 34) as well as attached to red corpuscles whose hemoglobin appears to be intact (Figs. 70 to 84).

The above stages of migration, when seen in one film, indicate that the process of migration is rapid and that the parasites do not long remain free. One would naturally expect an immediate attachment of the parasites when the surroundings are favorable to their growth, and no quinine or other injurious drug is present. I have seen parasites attaching themselves to fresh corpuscles before they had entirely abandoned the dehemoglobinized corpuscles.

Mannaberg [14] observed a tertian parasite (not a flagellating body) abandon a corpuscle, and he states: "It occurs with such rapidity and in such an unexpected manner that the details cannot be appreciated." In 1899, Dock[15] described what I believe to be migrating parasites. In several instances he saw free parasites. He pictures eight changes of form in a small, free, hyaline body, the observation lasting 5 minutes. He states that certain parasites seemed to have an especial tendency to escape from the blood corpuscles at early periods. And he seemed to be in doubt as to whether the free parasites were varieties of

[14] Mannaberg, J., The malarial parasites. A description based upon observations made by the author and by other observers, translation by Felkin, R. W., London, 1894, 344.

[15] Dock, G., *Med. News*, 1890, lvii, 61, 63.

the forms which carry on the typical process in malaria, or whether they had an independent existence. Bignami and Bastianelli[16] believed that, in the presence of a large number of free parasites, they had found evidence of an early destruction by decolorization of a large number of parasitiferous corpuscles. In 1914, Macfie,[17] in a case of æstivo-autumnal infection, observed an undoubted migration. He reports that a considerable number of free parasites were seen, many of which appeared to be young parasites which had not yet attached themselves. Other parasites, he states, were definitely ring-shaped, not infrequently possessing two chromatin masses. Undoubtedly the ring-shaped parasites and the parasites with the two chromatin masses had been recently attached to corpuscles and were freed in the form which they assumed when attached. The parasites with the two chromatin masses were two parasites, which had been attached to one corpuscular mound.

In studying migrations it will save time to work with the heavy infections.

Free Parasites.

Free parasites may be found in the blood of all malarial infections. In the æstivo-autumnal infections one may find free in the blood, round bodies and crescents, and parasites in the early stages of development. Instances of multiple infection of red corpuscles are more frequently seen in æstivo-autumnal infections than in other malarial infections. Where more than one parasite is attached to a corpuscle, they are set free prematurely by destroying the infected corpuscle sooner than one parasite would.

Free parasites may be (*a*) compact, (*b*) ring-form, or (*c*) ameboid, and they may show delicate filaments arising from the cytoplasm of the parasites for the purpose of attachment to fresh red corpuscles. Sometimes these filaments are so delicate as to be scarcely discernible and one's attention is first attracted to a pigment granule in connection with a filament so fine that otherwise it would not have been noticed.

Almost every worker who has done much research in connection with malarial parasites has seen parasites free, either in fresh or stained specimens. They have been so frequently seen and described that it seems surprising that a true interpretation of their presence in the blood did not suggest itself.

[16] Bignami, A., and Bastianelli, D. G., quoted from Thayer, W. S., Lectures on the malarial fevers, New York, 1897, 168.

[17] Macfie, J. W. S., *Lancet*, 1914, ii, 1354.

Laveran[18] writes of the parasites as "now free in the blood, now adherent to blood corpuscles." Mannaberg[19] has observed free not only "spores," but "parasites which are developed further than their spore-forming stage," and he states that the way their structure takes the stain is so striking that one is prevented from suspecting them to be degeneration forms, or from mistaking them for other blood elements. Canalis[20] has seen free parasites in æstivo-autumnal infections. Thayer[21] describes parasites as free in the cerebral capillaries. Marchiafava and Bignami[22] have observed free parasites. DaCosta[23] describes young parasites as escaping prematurely from red corpuscles. Ewing[24] states that, associated with intracellular parasites, he frequently saw young æstivo-autumnal parasites free in the plasma. Celli and Guarnieri[25] sketched the appearance of young extracellular bodies, including forms of at least two species of parasite. Gautier and Ziemann[26] have observed free parasites. Councilman and Abbott[27] described pigment-bearing bodies in and outside red corpuscles. Golgi[28] states that sometimes parasites may be seen free in the plasma. Ewing[29] must have attached a certain importance to the fact that free parasites were seen in the blood, for he has written of them under the heading of "Extracellular parasites."

The parasites illustrated in this article were secured from two cases of æstivo-autumnal infection.

[18] Laveran, A., Plaudism, translation by Martin, J. W., London, 1893, 12.

[19] Mannaberg, J., The malarial parasites. A description based upon observations made by the author and by other observers, translation by Felkin, R. W., London, 1894, 277.

[20] Canalis, P., quoted from Thayer, W. S., and Hewetson, J., *Johns Hopkins Hosp. Rep.*, 1895, v, 22.

[21] Thayer, W. S., Lectures on the malarial fevers, New York, 1897, 61.

[22] Marchiafava, E., and Bignami, A., in Stedman, T. L., Twentieth century practice, New York, 1900, xix, 291.

[23] DaCosta, J. C., Clinical hæmatology, Philadelphia, 1901, 365.

[24] Ewing, J., *J. Exp. Med.*, 1900–01, v, 446.

[25] Celli and Guarnieri, quoted from Ewing, J., *J. Exp. Med.*, 1900–01, v, 473.

[26] Gautier and Ziemann, quoted from Ewing, J., *J. Exp. Med.*, 1900–01, v, 473.

[27] Councilman and Abbott, quoted from Thayer, W. S., and Hewetson, J., *Johns Hopkins Hosp. Rep.*, 1895, v, 12.

[28] Golgi, quoted from Thayer, W. S., and Hewetson, J., *Johns Hopkins Hosp. Rep.*, 1895, v, 16.

[29] Ewing, J., *J. Exp. Med.*, 1900–01, v, 472.

SUMMARY.

The anemia in malarial infections is explained by the fact that each parasite destroys several red corpuscles.

Reduction of hemoglobin out of proportion to the loss of red corpuscles is explained by the fact that there is always a partial loss of hemoglobin in certain of the surviving corpuscles due to parasitic action.

Migration of parasites occurs in all æstivo-autumnal infections. If one wishes to observe migrations, it will save time to study the heavy infections.

Free parasites have been frequently noted by many observers, who have failed to interpret properly their presence in the blood.

EXPLANATION OF PLATES.

Plate 17.

ÆSTIVO-AUTUMNAL PARASITES.

Magnification, × 1,780.

Figs. 1 to 30. Free parasites in migration. The pigment granules (at o) are evidence of previous attachments.

Figs. 31 to 34. Young parasites on corpuscular skeletons. In Fig. 34 a pigment granule is shown at o.

Plate 18.

ÆSTIVO-AUTUMNAL PARASITES.

Magnification, × 1,780.

Figs. 35 to 37. Free parasites with attaching pseudopodia arising from the cytoplasm of the parasites. Pigment granules (at o) are evidence of previous attachments.

Fig. 38. Three young parasites freed from one corpuscle.

Figs. 39 to 41. Young parasites with attaching pseudopodia. Pigment granules indicating previous attachments may be seen at o. In Fig. 41 the filament connecting the pigment granule with the parasite is scarcely discernible.

Figs. 42 and 43. Instances of two young parasites freed from one corpuscle. In Fig. 42 a pigment granule is seen at o.

Figs. 44 to 58. Free ameboid young parasites. Pigment granules at o. Fig. 57 shows two free parasites; chromatin masses at x.

Fig. 59. Two free parasites; one is ameboid, the other at x is compact.

Figs. 60 to 63. Instances of two young parasites freed from one red corpuscle. Note that at o pigment granules may be seen in connection with the parasites.

Figs. 60, 62, and 63 show pigment granules in connection with but one of the parasites. In Fig. 63 the parasite at x is compact.

Fig. 64. Three young parasites probably freed from one red corpuscle. The two at x are compact.

Plate 19.

ÆSTIVO-AUTUMNAL PARASITES.

Magnification, $\times$ 1,780.

Fig. 65. A young parasite attached to a red corpuscle almost entirely dehemoglobinized, and an older parasite attached to a peripheral mound of a healthy appearing corpuscle. At x a young parasite is seen attached to a decolorized corpuscle.

Figs. 66 to 69. These figures show young parasites free and also instances of two parasites attached to one corpuscular mound (at x). Fig. 67 shows two pigment granules at o.

Figs. 70 to 79. Young parasites attached to fairly healthy appearing red corpuscles, the bodies of the parasites resting on the periphery of the corpuscle. Fig. 74 shows a pseudopodium arising from the cytoplasm of the parasite.

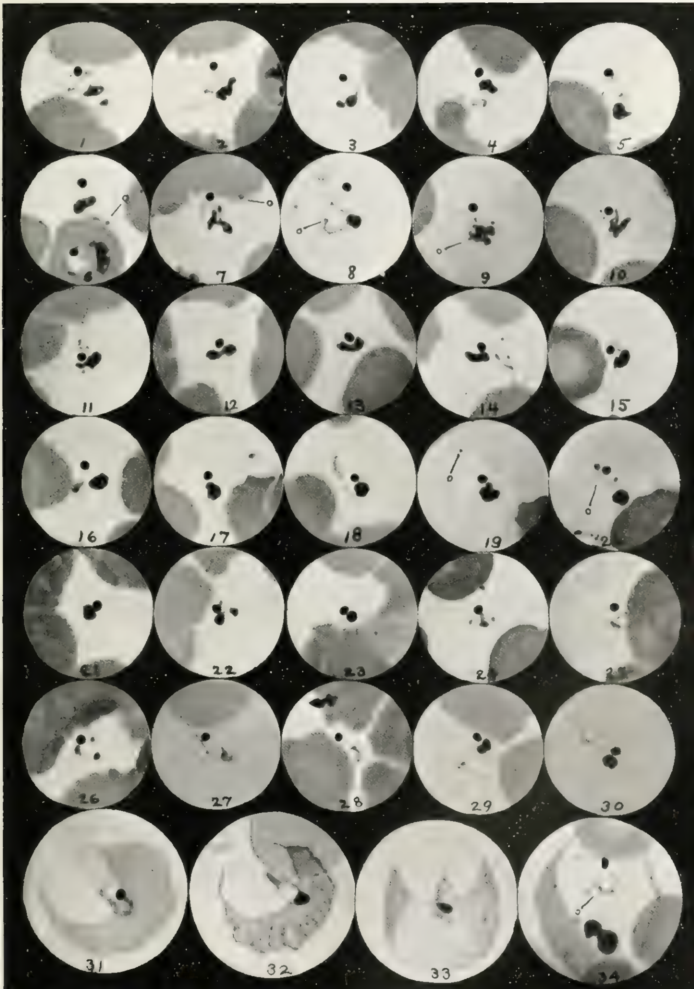
Figs. 80 to 84. Young parasites attached to peripheral corpuscular mounds of healthy appearing red corpuscles.

Fig. 85. A young parasite attached to a corpuscle by a delicate pseudopodium.

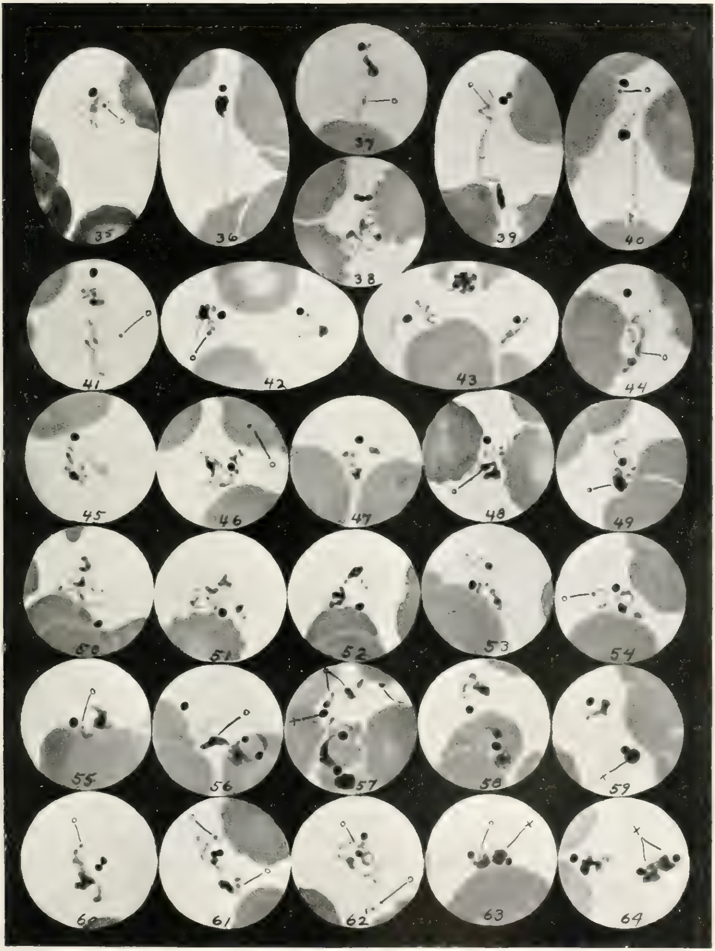
Figs. 86 to 91. Parasites partly off decolorized red corpuscles. Pigment granules may be seen at o. In Fig. 89 a compact parasite is seen at x. In Fig. 91 two young parasites are seen attached to a healthy appearing corpuscle, while one parasite has decolorized the corpuscle at their right.

Fig. 92. A young parasite attached to a peripheral mound of a decolorized red corpuscle. Part of the parasite extends beyond the periphery of the corpuscle. A pigment granule is seen at o.

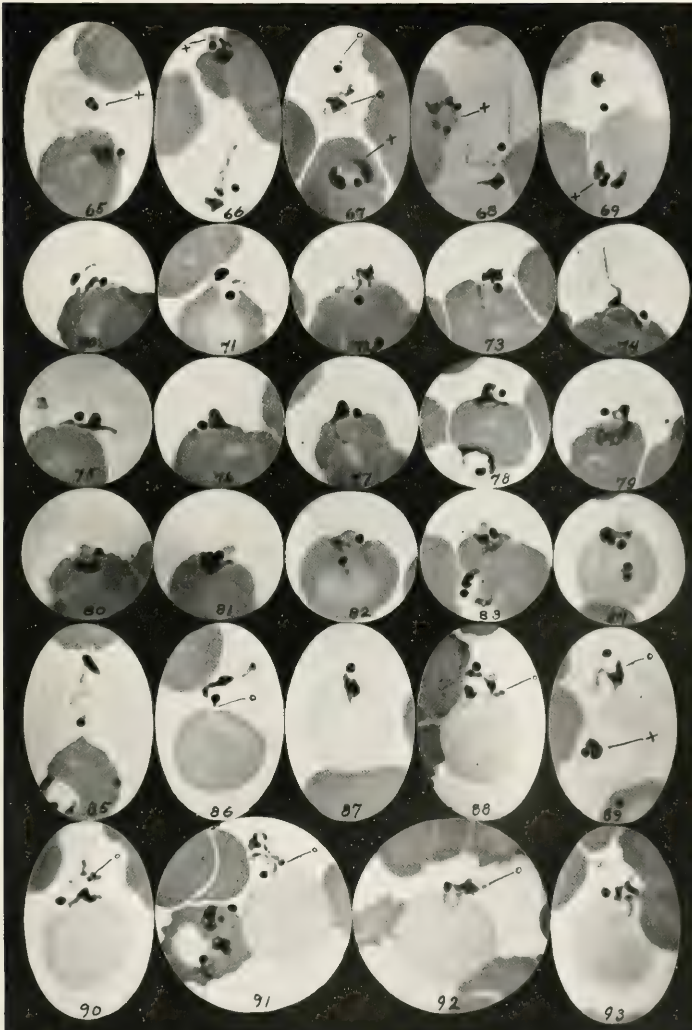
Fig. 93. A young parasite attached to a decolorized corpuscle.



(Lawson: Æstivo-autumnal malarial infections.)



(Lawson: Æstivo-autumnal malarial infections.)



(Lawson: Æstivo-autumnal malarial infections.)

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PERSISTENCE OF THE VIRUS OF POLIOMYELITIS IN THE NASOPHARYNX.

BY SIMON FLEXNER, M.D., AND HAROLD L. AMOSS, M.D

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, February 15, 1919.)

This paper is intended as a contribution to the epidemiology of poliomyelitis. Present knowledge places that disease among the infections in which the specific cause is carried in the nasopharynx, and present belief is to the effect that the virus is conveyed from one person to another through the medium of the nasopharyngeal secretions. Indeed, the virus has been detected in these secretions by the inoculation test in three sets of conditions; (1) coincidentally with an attack of the disease incited by it; (2) a considerable period after the attack of the acute disease has abated; and (3) in healthy persons who have been in contact with cases of poliomyelitis.

This determination having been made, detailed information covering the frequency with which and the conditions under which the virus can be discovered in the nasopharynx is greatly to be desired. This information is essential to the working out of the principles of the method of control of epidemic poliomyelitis.

Account must be taken, at the outset, of the difficulties surrounding, at the present time, the demonstration of the virus. The only reliable means of its detection is the inoculation test. When an active virus is injected intracerebrally or even otherwise into monkeys a train of symptoms tends to be set up in these animals which closely resembles the symptoms present in man, the subject of poliomyelitis. Indeed, the analogy is even closer than this, because the histological changes arising in the central nervous organs of the monkeys are an exact counterpart of those present in fatal cases of the disease in human beings. We can now state unreservedly that when typical symptoms appear in the inoculated monkeys typical lesions will occur in their nervous organs.

There is no division of opinion among observers regarding the clinical and histological evidences of poliomyelitis in monkeys in instances in which the experimental disease conforms to frank cases of poliomyelitis occurring in man. There is, however, lack of agreement respecting a less typical experimental condition described by Kling, Pettersson, and Wernstedt.[1] The questions raised by these experimenters, who have introduced entirely new criteria into the subject, are of far reaching significance, because they affect the character of the evidence acceptable as indicating the presence of the virus of poliomyelitis in the nasopharynx. This aspect of the general subject will come under consideration in connection with the experiments to be reported and discussed in the present paper.

Review of the Literature.

In order that the experiments to be described may take their proper place among the studies dealing with the mode of infection in poliomyelitis, a brief review of the literature, especially of that stressing the nasopharynx as the portal of entry and of exit of the virus, will be given.

That the virus, the existence of which at that time was merely suspected, is communicated by personal contact was the thesis which Wickman's[2] studies served to emphasize. But until Landsteiner and Popper[3] communicated the disease to monkeys no more precise definition of the mode of infection could be given. It will be recalled that Landsteiner and Popper originally conveyed the infection from man to monkey by the intraperitoneal route of inoculation and were thus able to reproduce poliomyelitis only in the first series of injected animals. The employment of the intracerebral route of inoculation by Flexner and Lewis[4] led to the discovery that the virus could be transmitted from monkey to monkey through an indefinite series, in course of which its activity or virulence for monkeys increased many fold. Moreover, still other portals of experimental infection were successively disclosed, such as the large nerves, subcutis, subarachnoid space, nasal mucosa, eye, and, although with far greater difficulty, the general blood.

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[1] Kling, C., Pettersson, A., and Wernstedt, W., *Communications Inst. méd. État Stockholm*, 1912, iii, 4.

[2] Wickman, I., Beiträge zur Kenntnis der Heine-Medinschen Krankheit, Berlin, 1907.

[3] Landsteiner, K., and Popper, E., *Z. Immunitätsforsch., Orig.*, 1909, ii, 377.

[4] Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1909, liii, 1639.

In addition, it was found by Leiner and von Wiesner[5] that the virus would penetrate the mucous membrane of the gastrointestinal tract of monkeys in which the motions of the digestive organs were for a time arrested by means of opium. It is noteworthy that aside from the intracerebral route the other modes of infection give more or less inconstant results, and that the external portal which is most favorable to the attack of the virus is the nasal mucous membranes.

The particularly favorable position afforded by the nasal route to infection is a fact the demonstration of which was indicated by circumstances surrounding the epidemiology of poliomyelitis in man. Flexner[6] pointed out that the not infrequent confusion of epidemics of poliomyelitis with those of cerebrospinal meningitis argued for a similarity not only in certain cardinal symptoms but also in the conditions under which the two diseases arise. Thus each disease attacked by preference infants and young children, although not wholly sparing older children and adults. Usually a single case appears in a family or home, but sometimes two cases, and less often three or more appear. These resemblances came to be further emphasized by the detection of the meningococcus carrier on the one hand and the demonstration of the ambulant and abortive cases of poliomyelitis on the other. Indeed, the single striking epidemiological point of difference relates to the seasonal prevalence which for poliomyelitis tends to be summer and autumn and for cerebrospinal meningitis winter and spring. Even this distinction is not absolute; we now know that winter epidemics of poliomyelitis occur, and circumstances favoring epidemics of cerebrospinal meningitis arise in the warm months of the year. The meningococcus carriers have been known for more than a decade; it is important to ascertain whether corresponding poliomyelitic virus carriers exist.

Experience having shown that a highly potent poliomyelitic virus may be secured by successive passages through monkeys, the way was opened for an experimental study of the nasopharynx as the portal of its entry into and exit from the body. The filterability of the virus, moreover, permitted its entire separation from the bacteria present in that locality. Flexner and Lewis,[7] therefore, crushed and extracted in salt solution the excised nasal mucous membrane removed from monkeys which succumbed to intracerebral inoculation of the virus and after filtration through a Berkefeld filter injected the fluid into other *rhesus* monkeys, thus inducing infection and paralysis. The corollary to this experiment, namely the infection of monkeys by direct inoculation of the virus into the nasal mucosa, was quickly supplied by Landsteiner and Levaditi.[8] The last test has been carried out successfully in several ways: by applying the virus to the abraded mucous membrane, by introducing it upon cotton tampons, and

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[5] Leiner, C., and von Wiesner, R., *Wien. klin. Woch.*, 1910, xxiii, 91.

[6] Flexner, S., *J. Am. Med. Assn.*, 1910, lv, 1105.

[7] Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1910, liv, 535.

[8] Landsteiner, K., and Levaditi, C., *Ann. Inst. Pasteur*, 1910, xxiv, 833.

finally by Leiner and von Wiesner[9] by merely pencilling the mucosa without causing any demonstrable lesion whatever. It should be stated, also, that one or the other of the modes of inoculation succeeds or not according, apparently, to the initial potency of the virus and the species of monkey chosen. Finally, some experimental evidence has been adduced to show that the nasal membranes are better suited to convey the virus than the tonsillar tissue. Levaditi and Danulesco[10] made submucous injections into the two regions and induced infection in the one case and not in the other.

The virus of poliomyelitis exists not only in the tissues but also in the secretions of the nasopharynx of monkeys, as shown by Landsteiner, Levaditi, and Danulesco,[11] who inserted cotton plugs into the nares of paralyzed animals and found that after several hours the absorbed fluid sufficed, when injected, to infect other monkeys; while Thomsen[12] ascertained also that merely rubbing the mucosa with the tampon was sufficient to incite infection. In this connection mention may be made of the fact that Landsteiner and Levaditi[8] consider that they have traced the passage of the virus along the olfactory nerves to the olfactory lobe of the brain, and Flexner and Clark[13] likewise have found the olfactory lobes infective, and the spinal cord and medulla non-infective, 48 hours after an intranasal inoculation of the virus. The last observation indicates that the penetration of the virus from the surface to the interior of the nasal membrane and thence to the brain occurs quickly, a point borne out by certain experiments made with antiseptic drugs to be reported elsewhere.

The foregoing experimental results provide a basis for considering the inoculations which have been made directly with human materials derived from the nasopharynx. These materials may be divided into three classes as follows: (1) washings, (2) tissues (tonsils and adenoids) removed during life, and (3) tissues (tonsils, pharyngeal mucosa, nasal mucosa) recovered after death. As regards the first, numerous tests have been made. It will be of doubtful value to note in this place the failure to induce infection. The negative results are, in the light of present knowledge, without real significance. The limits of activity for monkeys of virus immediately obtained from human beings are quickly reached. Hence the dilute filtered washings of the nasopharynx could be expected to succeed only rarely. We have ourselves failed to incite infection with a filtrate prepared from the spinal cord of human cases of poliomyelitis when the unfiltered suspensions were active.

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[9] Leiner, C., and von Wiesner, R., *Wien. klin. Woch.*, 1910, xxiii, 323.

[10] Levaditi, C., and Danulesco, V., *Compt. rend. Soc. biol.*, 1912, lxxii, 606.

[11] Landsteiner, Levaditi, and Danulesco, *Compt. rend. Soc. biol.*, 1911, lxxi, 558.

[12] Thomsen, O., *Berl. klin. Woch.*, 1912, xlix, 63.

[13] Flexner, S., and Clark, P. F., *Proc. Soc. Exp. Biol. and Med.*, 1912–13, x, 1.

However, Kling, Pettersson, and Wernstedt[1] have brought indubitable proof that washings obtained from the nasopharynx both during life from typical cases of poliomyelitis and after death from persons who succumbed to the disease, may carry the virus in a form and quantity capable of inciting infection in monkeys. If we confine ourselves strictly to the instances in which the washings conveyed typical experimental poliomyelitis, it may be said that while the number of failures to infect exceeds the successful inoculations, yet the latter comprise a convincing series. While opinion on this point can hardly be divided, the same cannot be stated for the interpretation which they place upon the results of the inoculation of washings from abortive cases, mere contacts, and from recovered persons, to which fuller reference will be made presently.

The first to detect the virus in tonsillar and pharyngeal mucosa obtained from a fatal case of poliomyelitis in man and thus to confirm Flexner and Lewis' experiments on the monkey were Landsteiner, Levaditi, and Pastia.[14] Flexner and Clark[15] about the same time reported several similar successful inoculations. The latter authors drew attention to the fact that when they injected filtrates of the tonsillar and nasal tissues infection failed; but when the same materials were rendered bacteria-free with 0.5 per cent phenol and inoculated as suspensions infection followed.

Thus far all the successful inoculations of the virus of poliomyelitis noted upon monkeys have been secured with materials obtained from recent typical cases of the disease in man or the experimental reproduction of that disease in those animals. An experimental answer to the important question of the period of the survival of the virus in the nasal and buccal mucosa was first successfully attempted by Lucas and Osgood,[16] who were still able to determine its presence in the experimentally infected monkey at the expiration of nearly 6 months.

Wickman's important studies[2] pointed unmistakably to the part played by so called abortive cases of poliomyelitis and healthy carriers of the microbic cause in disseminating poliomyelitis. The discovery of the experimental disease in monkeys led to the search for the virus in the nasopharynx of those two classes of persons. By far the most extensive inoculation tests published are those of Kling, Pettersson, and Wernstedt.[1] They even go beyond the phases of the subject indicated and include an investigation of the period of survival of the virus in the nasopharynx. Unfortunately this highly important study is marred by the fact that the criteria of the experimental disease in monkeys which the authors came to adopt do not conform to those which all experimenters accept as distinctive of poliomyelitis in the monkey. The point is an essential one. A close review of their protocols has led us to doubt the validity of their conclusions.

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[14] Landsteiner, K., Levaditi, C., and Pastia, C., *Semaine méd.*, 1911, xxxi, 296.

[15] Flexner, S., and Clark, P. F., *J. Am. Med. Assn.*, 1911, lvii, 1685.

[16] Lucas, W. P., and Osgood, R. B., *J. Am. Med. Assn.*, 1913, lx, 1611.

The inoculation experiments which form the basis of this paper deal with this aspect of the subject.

Meanwhile, it has been shown through inoculation in one instance by Flexner, Clark, and Fraser[17] that healthy persons may harbor the virus in their nasal and buccal secretions. The instance in which the demonstration was made related to the parents of a young child acutely ill with poliomyelitis. The contact, therefore, between the carriers and the case was an intimate one. This unquestioned observation was followed by a similarly conclusive one by Kling and Pettersson,[18] who, it may be remarked in passing, attribute failure to incite typical clinical and anatomical effects in their earlier experiments to the injections of insufficient amounts of the virus. Finally, Taylor and Amoss[19] have recorded two instances—one an abortive or non-paralytic case of poliomyelitis and the other a healthy child who subsequently developed frank paralysis—in which the concentrated nasopharyngeal washings inoculated into monkeys induced the typical experimental disease.

Nature of Materials and Interpretation of Experiments.

The experiments which we are reporting were made with human materials of two sorts; namely, tonsils and adenoids removed during life and tonsils and pharyngeal mucosa excised after death. The object in all the experiments was to ascertain the presence or absence of the virus as far as this could be determined by the inoculation test. In this way it was hoped to throw light on the persistence of the virus in the nasal and buccal membranes and their discharges.

The outcome of the tests is not so consistent as we would wish. But the inconsistency which will appear is instructive, for not only does it point the limitations of the inoculation test, but it helps clear up, at the same time, the more indefinite clinical effects which sometimes follow the injections of supposedly virus-containing materials. It may be mentioned here that we did not confirm the experiments of Kling, Pettersson, and Wernstedt respecting an atypical variety of poliomyelitis in the monkey which they finally came to attribute to the inoculation of too little virus-containing washings. Our tests, as will appear, were carried out with tissues and with quantities that should have sufficed for infection had the virus been present in amounts to be expected and in an active state.

[17] Flexner, S., Clark, P. F., and Fraser, F. R., *J. Am. Med. Assn.*, 1913, lx, 201.

[18] Kling, C., and Pettersson, A., *Deutsch. med. Woch.*, 1914, xl, 320.

[19] Taylor, E., and Amoss, H. L., *J. Exp. Med.*, 1917, xxvi, 745.

The last considerations are pertinent because of the striking contrast in the results obtained with human tissues removed either by operation or post mortem. The obvious deduction is to the effect that the upper respiratory mucosa carries far more active virus in the latter instance. All the postmortem tissues inoculated by us were derived from acute cases. Analogy with other acute infections leads to the assumption that in the fatal instances there is more active multiplication of the virus than in cases which go on to recovery. This is perhaps the simplest interpretation to put upon our tests.

The discrepancy seems to illuminate the question as to the time of maximum infectivity in epidemic poliomyelitis. As far as observations made during the great epidemic, which prevailed in New York State and elsewhere in 1916, can be construed, the implication is to the effect that this period is relatively brief and is greatest early in the disease. It cannot be said, however, that this point is definitely established. As a matter of fact, the literature contains records which directly controvert this idea. The matter is perhaps one not to be settled absolutely but rather to be placed on a relative basis. What we need first to know are the conditions under which the virus of poliomyelitis can be definitely determined to be present in the nasopharynx. This question we have endeavored to answer by a study of tissues removed post mortem and of hypertrophied tonsils and adenoids extirpated during life from persons who had suffered a typical attack of poliomyelitis some weeks or months earlier. In comparing the two classes of tissue, attention must be given the fact that the former, that is the materials removed post mortem, come from cases earlier in the course of the disease.

The results of the inoculation of the tonsillar and adenoid tissues removed during life have also an important bearing on the question of the chronic carriage of the virus upon the nasopharynx. The test of Lucas and Osgood[16] showed that the virus may be demonstrable on the nasopharyngeal mucous membrane of the monkey as long as 5 months after infection had been experimentally induced. Flexner and Clark[20] demonstrated it in the nasopharyngeal mucosa of a monkey surviving the paralysis more than 4 weeks and failed, in the

[20] Flexner, S., and Clark, P. F., *J. Am. Med. Assn.*, 1911, lvi, 585.

same animal, to detect it in the spinal cord. The large series of tests with washings from human cases made by Kling, Pettersson, and Wernstedt[1] on the basis of which they believe they have shown chronic carriage of the virus to be common, have never been subjected to a searching control with human tissues. Our experiments with surgically removed tonsils and adenoids may be regarded as covering this point. It happens, indeed, that our results are quite opposed to those of the Swedish observers and teach rather that the chronic carriage is, at least, exceptional. On the whole the epidemiological data are in conformity with our experimental results.

The Swedish authors studied nine convalescents over a maximum period of 7 months on the basis of which they formulated the following deductions: "that the secretion from the mucous membranes of the mouth and intestine of persons who have recovered (from poliomyelitis) has had the power of infecting monkeys still several months, in one case 204 days (nearly 7 months), after the onset of the illness, giving rise to an experimental poliomyelitis with fatal issue . . . . It was only in one case that we did not succeed in demonstrating the presence of the virus after the comparatively short time of 30 days."[21]

In interpreting this statement and in considering the discrepancy with our studies, it is necessary to take into account the immediately succeeding paragraphs:

"During the time occupied by the investigations, the virus had changed its character, so that it no longer caused inflammations with cellular exudations. Instead of this the degeneration of the nerve cells, the changes of the glia cells and the neurophagocytosis caused by the enlarged glia cells have been the characteristic changes. They have thus been of the same type as those appearing in the monkeys injected with secretions from abortive cases, and virus carriers, changes which . . . . we consider ourselves justified in assuming to be due to a less virulent virus. *The experiment also shows, that the microbe rather quickly—already after 8–14 days—loses its power of causing inflammatory exudations in the inoculated animals. This fact is of very great importance from a practical point of view since it perhaps gives us the right to assume that the virus, possibly rather soon after the termination of the acute stage, gets weaker.*"[22] (Author's italics.)

We propose now to present our results in the form of tabulations with such discussion as seems called for. The postmortem tissues

[21] Kling, Pettersson, and Wernstedt,[1] p. 159.

[22] Kling, Pettersson, and Wernstedt,[1] pp. 159–160.

employed for inoculation were obtained very soon after the death of the patient, when they were either inoculated after a short interval, or placed in the preserving fluids, which consisted of 50 per cent sterile glycerol or 0.5 per cent phenol. The tissues removed surgically were put immediately into the preservative fluids. The inoculations were made at leisure. The glycerolated or phenolized specimens were suspended and injected partly intracerebrally and partly intraperitoneally. Previous experiments had shown that the weak phenol destroyed associated bacteria in the tissues without acting appreciably upon the virus of poliomyelitis.[15] In this respect the phenol was superior to the glycerol, which also was without observed injurious action on the virus, but which removed the bacteria much more slowly.

Histological studies were made with the nervous organs of all monkeys succumbing to or after the inoculations. All the animals which developed typically clinical poliomyelitis showed characteristic histological lesions in the spinal cord and medulla and intervertebral ganglia. In none of the animals in which the clinical symptoms were dubious did we find histological lesions either resembling those of typical poliomyelitis or corresponding with the degenerative and peculiar neurophagocytic ones which Kling, Pettersson, and Wernstedt[1] describe and attribute to the action of specifically weakened poliomyelitic virus.

Examination of Table I brings out the fact that both the tonsils and nasal mucosa of fatal early cases of poliomyelitis are infectious for monkeys. No real distinction can be drawn between the two sets of materials as regards their infectivity. The fact that now one tissue from an individual succeeds while the other fails is probably as ascribable to differences in the susceptibility of the individual monkeys as to irregularity in distribution of the virus. The inferiority of filtrates to emulsions or suspensions of the tissues is manifest. It is clear also that phenol is a favorable medium for preparation of the tissues for inoculation, as it tends quickly to destroy bacteria associated with the virus without materially injuring the virus itself. Probably the 0.5 per cent phenol is only relatively innocuous for the virus, since in the instance of H. K., in which two *rhesus* monkeys were inoculated,

TABLE I.

Human Tissues Obtained post Mortem.

Case.	Day of disease.	Monkeys inoculated.	Material injected.	Symptoms.	Histology.	Remarks.
J. A. R.	6	2 *M. rhesus.*	Berkefeld filtrate of tonsils and pharynx mucosa.	None.		Filtrate of spinal cord negative; emulsion positive.
R. P.	7	2 *M.* "	(1) Emulsion of tonsils.	Tonsils none.	Typical lesions from nasal mucosa.	Glycerol preservation. Nasal mucosa positive Tonsils negative.
			(2) Emulsion of nasal mucosa.	Nasal mucosa typical.		
M. K.	6	2 *M* "	Heim filtrate of tonsils and nasal mucosa separately.	None.		Glycerol preservation.
J. C.	3	1 *M.* " 1 *M. cynomolgus.*	Heim filtrate of tonsils and nasal mucosa separately.	Nasal mucosa none. Tonsils typical.	Typical lesions from tonsils.	Glycerol preservation. Tonsils positive in *M. cynomolgus.* Nasal mucosa negative.
B. T.	6	1 *M. rhesus.*	Emulsion of tonsils.	Typical.	Typical lesions.	Phenol preservation. Reinoculation of spinal cord positive.
J. G.	7	1 *M.* "	" " "	"	" "	Phenol preservation.
S. A.	9	2 *M.* "	" " " and pharynx mucosa separately.	None.		" "

H. K.	6	2 *M. rhesus*	Emulsion of tonsils	Typical, with recovery.		Phenol preservation in successive quantities to remove resistant bacteria.
G. G.	6	3 *M.* "	" " "	Typical.	Typical lesions.	Phenol preservation.
A. K.	8	1 *M.* "	" " "	None.		
J. W.	4	1 *M.* "	" " " and pharynx mucosa.	"		
G. C.	?	1 *M.* "	Heim filtrate of tonsils.	Indefinite.	Tuberculosis	
B. H.	10	1 *M.* "	Heim filtrate of tonsils.	None.		
A. S.	3	1 *M.* "	Emulsion of tonsils intrasciatic and intraperitoneal.	"		Glycerol preservation.

TABLE II.

Human Tissues Removed Surgically.

Case.	Day of disease.	Monkeys inoculated.	Material injected.	Symptoms.	Histology	Remarks.
H. G.	8	1 *M. rhesus.*	Berkefeld filtrate of tonsils and adenoids.	None.		
I. P.	13	1 *M.* "	Emulsion of tonsils and adenoids.	Gradual emaciation with diarrhea. Died 15th day.	No lesions in nervous organs.	Phenol preservation.
A. S.	13	1 *M.* "	Heim filtrate of tonsils and adenoids.	Indefinite. Recovered.		
J. P.	13	1 *M.* "	Emulsion of tonsils and adenoids.	None.		Glycerol and phenol preservation.
H. B.	14	1 *M.* "	Emulsion of tonsils and adenoids.	Emaciation. Died 15th day.	Tuberculosis.	Phenol preservation.
C. M.	17	1 *M.* "	Filtrate of tonsils and adenoids.	None.		" "
H. N.	18	2 *M.* "	Emulsion of tonsils and adenoids.	"		
C. U.	19	2 *M.* "	Berkefeld filtrate of tonsils and adenoids.	"		
J. A.	19	2 *M.* "	Emulsion of tonsils and adenoids	Indefinite.	Tubercles in spinal cord.	Glycerol preservation. Succumbed to tuberculosis.
F. S.	22	1 *M.* "	Berkefeld filtrate of tonsils and adenoids.	None.		

J. W.	25	2 *M. rhesus.*	Emulsion of tonsils and adenoids.	None.		Glycerol preservation.
H. F.	25	2 *M.* "	Emulsion of tonsils and adenoids.	(1) None. (2) Pressure. Symptoms from which recovered. Then indefinite. Death on 12th day.	No lesions suggestive of poliomyelitis. No degeneration of nerve cells in medulla, cord, or intervertebral ganglia, and no cellular infiltration.	Glycerol preservation. Small sterile cyst at point of inoculation.
F. S.	27	2 *M.* "	Emulsion of tonsils and adenoids.	None.		Glycerol preservation.
L. F.	27	2 *M.* "	Emulsion of tonsils and adenoids.	"		Glycerol preservation.
A. L.	27	1 *M. cynomolgus.*	Emulsion of tonsils and adenoids.	"		Phenol preservation.
C. J.	28	1 *M. rhesus.*	Filtrate of tonsils and adenoids.	"		
H. F. S.	28	2 *M.* "	Berkefeld filtrate of tonsils and adenoids.	"		
A. S.	35	1 *M.* "	Berkefeld filtrate of tonsils.	"		
G. C.	35	1 *M.* "	Emulsion of tonsils and adenoids.	"		Phenol preservation.
T. P.	39	1 *M.* "	Filtrate of tonsils and adenoids.	Indefinite. Recovered.		" "

TABLE II—*Concluded.*

Case.	Day of disease.	Monkeys inoculated.	Material injected.	Symptoms.	Histology.	Remarks.
J. O'B.	49	2 *M. rhesus.*	Emulsion of tonsils and adenoids.	(1) Died suddenly 8th day. (2) Gradually lost strength. Died 15th day.	(1) No lesions in nervous organs. (2) No lesions in nervous organs.	Glycerol preservation.
D. M.	50	2 *M.* "	Emulsion of tonsils and adenoids.	(1) None (2) Indefinite.	(2) No lesions in nervous organs.	Glycerol preservation 5 days. Cyst in cerebrum at point of inoculation.
F. McK.	50	2 *M.* "	Emulsion of tonsils and adenoids.	(1) Typical. Died 41st day. (2) None.	(1) No lesions in nervous organs.	Phenol preservation.
T. M.	90	1 *M.* "	Emulsion of tonsils and adenoids.	None.		Phenol preservation. Reinoculation with emulsion.
J. W.	90	1 *M.* "	Emulsion of tonsils and adenoids.	Died suddenly 2½ mos. after 1st and 19 days after 2nd inoculation.	No lesions in central nervous organs.	Phenol preservation. Reinoculation with emulsion.
M. K.	120	1 *M.* "	Emulsion of tonsils and adenoids.	Indefinite after 2nd inoculation. Recovered.		Phenol preservation. Reinoculation with emulsion.
E. S.	150	1 *M.* "	Emulsion of tonsils and adenoids.	Indefinite after 2nd inoculation. Recovered.		Phenol preservation. Reinoculation with emulsion.

and in which the tissues employed were put through three successive solutions of the phenol to render them approximately bacteria-free, a milder form of poliomyelitis developed. The number of tests made is insufficient to account for certain obvious discrepancies as, for example, the failure of tissues derived from cases dying on the 3rd and 4th day of the disease to infect. Doubtless the fact that only one monkey was used in each instance for inoculation had something to do with the failures. But at times the supply of monkeys was too small and precarious to permit of more being tested. Experience with inoculations of the spinal cord and medulla from human cases has shown us that the failure to incite infection in a single *rhesus* monkey does not indicate lack of power to infect still other individual animals of the same species.

The deduction from the tests summarized in the table is to the effect that the nasal and pharyngeal mucosæ of persons succumbing to poliomyelitis during the 1st week or 10 days of the disease probably regularly contain the poliomyelitic virus.

A glance at Table II shows at once a fundamental difference in the results of inoculating the specimens removed surgically and those obtained at autopsy. The extent of the distinction is not greatly lessened by the probability that the tests carried out with the surgical tissues err on the side of negativity. That certain of the surgical specimens contained virus in some amount we think most probable. But the essential fact remains that under the conditions of the experiments they regularly failed to incite infection and paralysis in the monkeys.

The two sets of tissues, those removed post mortem and those removed during life, differed especially in one respect; namely, that the former came from cases of poliomyelitis in the 1st week and the latter later in the course of the disease. We are inclined to attribute to this circumstance, with which may be associated the tendency for microorganisms to multiply more freely in the last hours of life, the great differences in effects observed.

In order to favor the induction of infection with the specimens removed relatively late after recovery from the acute symptoms of poliomyelitis, the reinoculation method of Flexner, Noguchi, and

Amoss[23] was also employed. By this method it is possible to convert a subminimal infective dose of the virus into an effective dose. But still no success was achieved with the surgical tissues.

DISCUSSION.

The epidemiology of poliomyelitis has still to be worked out in detail, as many factors governing the spread of the disease remain to be discovered. That the virus or microbic cause is communicated by personal contact is now generally admitted. That the virus occurs in the nasopharynx, which constitutes the chief locus of ingress and egress to and from the body is also conceded. The fact that the virus has been, if rarely, detected in healthy persons who have been in intimate contact with early cases of poliomyelitis, and even in certain individuals who have recovered from the acute effects of the disease, has led to the generalization that like some other diseases of bacterial origin, and notably epidemic meningitis, healthy and chronic carriers of the virus are frequent. This view has received its main support from Kling, Pettersson, and Wernstedt, whose studies we have discussed. A critical analysis of the basis of their contention fails, however, to carry conviction, and the doubt which has arisen as to the true interpretation of their results is deepened, we think, by our more searching tests.

The results of the experiments reported in this paper conform closely with clinical experience in the United States, at least, and especially with the observations made by epidemiologists in the course of the wide epidemic in New York State and elsewhere during the summer and autumn of 1916. The conclusion reached at that time was to the effect that the communicability of the disease was a phenomenon chiefly of the early stages, while the frankly paralyzed person and the convalescent were to be feared much less. In our experiments infection was secured with tissues obtained during the 1st week, approximately, of the disease but not at the later periods.

CONCLUSIONS.

The virus of poliomyelitis occurs in the nasopharynx of man and monkeys.

[23] Flexner, S., Noguchi, H., and Amoss, H. L., *J. Exp. Med.*, 1915, xxi, 91.

In man it has been detected by the inoculation test in washings from acute cases, rarely in similar washings from healthy contacts, in the nasopharyngeal tissues obtained from fatal cases in the 1st week of infection, but rarely, if ever, from nasopharyngeal tissues removed surgically at later periods in the course of the disease.

In monkeys, also, the virus has been detected in the secretions from acute experimental infections, in the nasopharyngeal tissues derived from early cases, and rarely from cases several weeks or months after recovery from the acute symptoms.

The inoculation of tonsils and adenoids obtained from cases of undoubted poliomyelitis either yielded definite results in the form of typical paralysis and histological lesions in the central nervous organs of the monkeys injected, or no symptoms or lesions which could be confounded with poliomyelitis. The indefinite symptoms and atypical lesions described in a certain class of inoculated animals by Kling, Pettersson, and Wernstedt were not encountered in our experiments.

The deduction from the experiments reported is to the effect that the virus is regularly present in the nasopharynx in cases of poliomyelitis in the first days of illness, and especially in fatal cases; that it diminishes relatively quickly as the disease progresses, except in rare instances; and that it is unusual for a carrier state to be developed. Hence the period of greatest infectivity of patients would appear to be early in the disease, which is probably the time at which communication of the virus from person to person takes place.

Available evidence proves that healthy carriers of the virus occur. We do not, however, possess data which indicate the frequency with which carriage arises. The fact that even after a severe and wide epidemic, such as occurred in the United States in 1916, the disease may virtually disappear within 2 or 3 years, points to the probability that enduring carriers of the active virus, whether healthy or chronic, are of exceptional occurrence.

We are greatly indebted to Dr. Cornelius G. Coakley for many of the specimens of surgically removed tonsils and adenoids with which our experiments were performed. We desire to express sincere appreciation for his cordial cooperation.

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EFFECT OF BILE ON THE CLOTTING TIME OF BLOOD.

BY HERBERT HAESSLER, M.D., AND MARIANNE G. STEBBINS.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Although it is known that jaundice tends to cause delayed clotting of blood, the cause of the delay is not known. Minot and his associates,[1] using Howell's method of recalcifying oxalated plasma, found that the coagulation time (prothrombin time of Howell) was increased in a series of jaundice cases, but did not suggest an explanation of the mechanism of the delay in coagulation. It seemed of interest, therefore, to determine whether or not bile or bile salts, which are, of course, present in the blood in jaundice, are in themselves capable of causing the increase in the coagulation time.

Effect of Bile on Clotting of Plasma.

Series 1.—Cats, under ether anesthesia, were bled from a large artery, through a paraffined cannula into paraffined 50 cc. centrifuge tubes containing 7.5 cc. of 1 per cent sodium oxalate in 0.9 per cent sodium chloride solution. The tubes were then centrifuged and the plasma was carefully pipetted off. If the plasma showed the least trace of hemolysis it was rejected. A series of flat bottom tubes, 22 mm. in diameter, was then set up, each containing 2 cc. of plasma and 0.5 cc. of an ox bile solution of varying concentration. To each of the tubes an amount of calcium chloride was added which had been previously found to produce a firm clot in the minimum time with the same plasma. The time necessary for the formation of a firm clot in each tube was recorded. Precipitation of fibrin was considered complete when a clot of such consistency was formed that the tubes could be inverted without loss of liquid. As shown in Table I, clotting time was delayed in proportion to the amount of bile present

[1] Minot, G. R., Denny, G. P., and Davis, D., *Arch. Int. Med.*, 1916, xvii, 101.

TABLE I.

2 cc. of oxalated plasma plus 0.5 cc. of bile solution, in dilution to give the final concentration indicated, were recalcified with the optimum quantity of calcium chloride solution.

Bile.	Clotting time.	Bile.	Clotting time.
per cent	*min.*	*per cent*	*min.*
9.0	No clot.	4.0	7
8.0	" "	3.3	4
7.7	" "	2.5	4
7.4	52	2.0	4
6.9	32	1.0	5
6.6	27	0.0	3
5.0	14		

in the plasma. The results were uniform with all the animals in this series.

Similar results were obtained when whole oxalated blood was used to which varying quantities of bile had been added.

Effect of Sodium Glycocholate on Clotting of Plasma.

Series 2.—In these experiments a solution of sodium glycocholate was substituted for bile. The experiments were otherwise identical with those of Series 1. Table II, the record of a typical experiment of this series, shows that results are similar to those of the first set.

We may conclude that bile and bile salts, in sufficient quantity, retard the coagulation of blood. Most modern workers conceive of the process of coagulation as taking place in two steps: first, the formation of thrombin; and second, the conversion of fibrinogen into

TABLE II.

0.5 cc. of oxalated plasma, containing the indicated concentration of glycocholate, was recalcified with the optimum quantity of calcium chloride solution.

Sodium glycocholate.	Clotting time.	Sodium glycocholate.	Clotting time.
per cent	*min.*	*per cent*	*min.*
0.5	13	0.2	5
0.4	6	0.1	5
0.3	5	0.0	5

fibrin by the action of thrombin. To determine whether the bile salts prevented the formation of thrombin or merely interfered with the change of fibrinogen to fibrin even though an adequate supply of thrombin was present, the effect of bile and bile salts on the clotting time of a solution of fibrinogen in the presence of sufficient quantities of preformed thrombin was tested in Series 3 and 4. Solutions of fibrin and thrombin were prepared according to the method of Mellanby.[2] Not only is this method simple and rapid, but the other clotting elements which are present in small amounts do not interfere.

Preparation of Fibrinogen.—Fowl plasma was used because it may be kept for weeks without spontaneous clotting. Fibrinogen was precipitated out by diluting the plasma with ten volumes of distilled water and neutralizing with a few drops of 1 per cent acetic acid. The precipitate was collected and dissolved in 0.5 per cent sodium chloride solution, about 40 cc. being a convenient amount when 50 cc. of plasma were originally used. Since this solution clotted upon addition of both thrombokinase and calcium, but not upon addition of either alone, it was concluded that it contained fibrinogen and prothrombin.

Preparation of Thrombin.—10 cc. of the above solution were coagulated by the addition of a trace of thrombokinase (extract of chick embryo) and calcium chloride to a concentration of 0.05 per cent. After removal of fibrin, the clear, residual fluid contains a quantity of thrombin proportional to the strength of the original fibrinogen-prothrombin solution, and a trace of thrombokinase. It is not essential to have thrombin free of other elements, provided that sufficient thrombin is available in solution and that there are no substances present which interfere with its action.

Effect of Bile and Bile Salts on Clotting of Fibrinogen in the Presence of Thrombin.

Series 3.—In these experiments 0.5 cc. of a solution of fibrinogen was used, to which bile had been added to the desired concentration. An excess of thrombin was then added and the clotting time noted. The result of one such experiment is recorded in Table III.

[2] Mellanby, J., *J. Physiol.*, 1917, li, 396.

Series 4.—Experiments in this series were carried out in the same way, except that sodium glycocholate was substituted for ox bile (Table IV).

TABLE III.

0.5 cc. of fibrinogen solution plus enough bile to make the indicated final concentration.

Bile.	Clotting time.	Bile.	Clotting time.
per cent	*min.*	*per cent*	*min.*
13	No clot.	6	15
11	Loose clot.	5	7
9	" "	4	2
7	21	3	2

TABLE IV.

0.5 cc. of fibrinogen solution plus sodium glycocholate.

Sodium glycocholate.	Clotting time.	Sodium glycocholate.	Clotting time.
per cent	*min.*	*per cent*	*min.*
1.2	No clot.	0.6	7
1.0	Loose clot.	0.5	4
0.8	14	0.4	3
0.7	10	0.0	4

Effect of Sodium Oleate on Clotting of Fibrinogen in the Presence of Thrombin.—A few experiments were also performed with sodium oleate instead of bile salts. Sodium oleate, which is the salt of an unsaturated fatty acid, is somewhat similar to sodium glycocholate in physical properties. Small amounts did not interfere with the clotting time; very large amounts inhibited clotting entirely; and moderate amounts caused delayed clotting. In no experiment, however, was there the same gradual increase in clotting time with increase of the salt which was observed in experiments with bile or sodium glycocholate. The superficial resemblances of the results, however, suggested that the mechanism may be the same as in the case of bile salts.

DISCUSSION.

It is evident from the above experiments that within certain limits clotting time depends on the percentage of bile present in solution and that the reaction is the same in experiments with pure solutions

of the substances concerned in coagulation, as in whole plasma. It likewise seems justifiable to conclude that bile and bile salts do not interfere with the formation of thrombin, since the prolongation of clotting time is just as great when preformed thrombin is added in ample quantity to fibrinogen solution, as when thrombin must be formed from its precursors in the presence of bile. It cannot be a question of destruction of the thrombin, as Morawitz and Bierich[3] showed that a quantity of freshly drawn blood which had been mixed with enough bile to inhibit clotting, could be caused to coagulate by merely diluting the mixture with isotonic salt solution. Consequently we must assume that it is the conversion of fibrinogen to fibrin that is interfered with rather than the formation of thrombin.

In our experiments it was found that there was a retardation of clotting, great enough to be detected by clinical methods, with amounts of bile greater than 5 per cent. We were unable to find reports in the literature stating the exact amounts of bile salts present in the blood in jaundice. Gilbert[4] states that in cases of obstructive jaundice bile pigment is present in the blood in quantities of from 0.7 to 1 gm. per liter. Bile itself contains about 1 gm. of pigment per liter. The relation of bile pigments and bile salts in the blood in jaundice has not been determined, but it would seem possible for the salt to be present in sufficient concentration to prevent clotting.

CONCLUSIONS.

1. Within certain limits the clotting time of blood, of blood plasma, and of solutions of fibrin to which bile salts have been added, is proportional to the quantity of bile present.

2. The bile interferes with the conversion of fibrinogen into fibrin and not with the formation of thrombin.

[3] Morawitz, P., and Bierich, R., *Arch. exp. Path. u. Pharmakol.*, 1907, lvi, 115.

[4] Gilbert, quoted by Wells, H. G., Chemical Pathology, Philadelphia and London, 3rd edition, 1918, 486.

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A FUNCTIONAL AND PATHOLOGICAL STUDY OF THE CHRONIC NEPHROPATHY INDUCED IN THE DOG BY URANIUM NITRATE.*

BY WILLIAM DEB. MACNIDER, M.D.

(*From the Laboratory of Pharmacology of the University of North Carolina, Chapel Hill.*)

PLATES 26 TO 30.

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Since the initial observation in 1888 by Chittenden and Hutchinson[1] that uranium salts would induce an acute nephropathy, these substances have been extensively used as acute nephrotoxic agents. Only a few observations have been made of the effect of these salts on the kidney in prolonged intoxications. Such observations as have been made are largely concerned with the type of the pathological response on the part of the kidney, and a study of the processes of repair which take place in the kidney during its recovery from the acute injury.

In 1904 Richter[2] employed uranium to induce a condition in animals analogous to Bright's disease. As a result of Richter's observations, uranium was employed by Dickson[3,4] in two researches, in which he demonstrated that prolonged intoxications by uranium nitrate would induce in guinea pigs, rabbits, and dogs a kidney injury which was comparable with some of the types of chronic kidney disease found in man. The form of injury most frequently induced was

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[1] Chittenden, R. H., and Hutchinson, M. T., Some experiments on the physiological action of uranium salts, *Tr. Conn. Acad. Arts and Sc.*, 1888–92, viii, 1.

[2] Richter, P. F., Die experimentelle Erzeugung von Hydrops bei Nephritis, *Beitr. klin. Med.*, Festschrift, Professor Senator, Berlin, 1904, 283.

[3] Dickson, E. C., A report on the experimental production of chronic nephritis in animals by the use of uranium nitrate, *Arch. Int. Med.*, 1909, iii, 375.

[4] Dickson, E. C., A further report on the production of experimental chronic nephritis in animals by the administration of uranium nitrate, *Arch. Int. Med.*, 1912, ix, 557.

of a diffuse interstitial type which in some instances went to the stage of granular atrophy. Associated with these later stages, certain of the animals became polyuric.

The present investigation has been undertaken with the object in view of studying the functional capacity of the kidney during the period of acute injury from uranium, and also during the period when the kidney is recovering from the acute degeneration and passing into that stage of chronic injury which is characterized by such changes in structure that the kidneys may be considered to represent some type of chronic nephropathy. The investigation has a further object. In previous papers[5,6] the observation has been made that the acute injury to the normal kidney, or to the naturally nephropathic kidney, by uranium is associated with the development of an acid intoxication. In the present study observations will be made of the changes in the acid-base equilibrium of the blood of these animals, not only during the period of acute damage to the kidney, but during the period of recovery from such an injury, when the relation may be studied of such changes in the blood with the processes of repair in the kidney, and the return of the functional response of the kidney.

EXPERIMENTAL.

Twenty-seven female dogs have been used in this series of experiments. The animals have varied from 5 months to $10\frac{1}{2}$ years in age. They were kept in metabolism cages for 4 days prior to the commencement of the intoxication. During this period studies of the urine, blood, and the functional capacity of the kidney were made, in order to eliminate animals with a naturally acquired nephropathy. All the animals were free from renal disease. The animals were given 500 cc. of water daily, and fed on bread with

[5] MacNider, W. deB., The inhibition of the toxicity of uranium nitrate by sodium carbonate, and the protection of the kidney acutely nephropathic from uranium from the toxic action of an anesthetic by sodium carbonate, *J. Exp. Med.*, 1916, xxiii, 171.

[6] MacNider, W. deB., The stability of the acid-base equilibrium of the blood in naturally nephropathic animals, and the effect on renal function of changes in this equilibrium. I, *J. Exp. Med.*, 1918, xxviii, 501.

a small amount of cooked lean beef. The animals were catheterized once a day, and the amount of urine obtained was added to the cage urine for analysis. The experiments were terminated at various periods during the intoxication without employing an anesthetic. This method of termination eliminates the probability of inducing acute degenerative changes in the liver as well as the kidney. The animals were poisoned by one dose of 4 mg. of uranium nitrate per kilo. The uranium was given subcutaneously.

During the course of the experiments the urine was examined quantitatively for albumin by Esbach's method, and for glucose with Benedict's reagent. The functional capacity of the kidney was studied by noting the percentage retention of blood urea, as shown by Marshall's[7] method, as modified by Van Slyke and Cullen,[8] and also by the elimination of phenolsulfonephthalein. The latter functional test was conducted according to the technique of Rowntree and Geraghty.[9] Observations on the acid-base equilibrium of the blood were made by employing the methods of Marriott[10,11] by ascertaining the alkali reserve of the blood and the tension of alveolar air carbon dioxide.

The Course of Prolonged Intoxication by Uranium in the Dog.

A large number of preliminary experiments has been necessary, in order to determine the dose of uranium which would effect a severe renal injury, and yet not be sufficiently toxic to injure the kidney so that processes of repair could not arise and lead to the development of a chronic nephropathy. As a result of these pre-

[7] Marshall, E. K., Jr., A rapid clinical method for the estimation of urea in urine, *J. Biol. Chem.*, 1913, xiv, 283.

[8] Van Slyke, D. D., and Cullen, G. E., A permanent preparation of urease, and its use in the determination of urea, *J. Biol. Chem.*, 1914, xix 211.

[9] Rowntree, L. G., and Geraghty, J. T., An experimental and clinical study of the functional activity of the kidneys by means of phenolsulphonephthalein, *J. Pharmacol. and Exp. Therap.*, 1909–10, i, 579.

[10] Marriott, W. McK., A method for the determination of the alkali reserve of the blood plasma, *Arch. Int. Med.*, 1916, xvii, 840.

[11] Marriott, W. McK., The determination of alveolar carbon dioxide tension by a simple method, *J. Am. Med. Assn.*, 1916, lxvi, 1594.

liminary experiments, an observation previously made by me[12] was confirmed, that the toxicity of uranium, as expressed by the severity of the kidney injury, largely depended upon the age of the animal.

A study of the experiments which follow not only illustrates the relative toxicity of uranium for animals of different ages, but also shows that young animals may so repair the damage done the kidney by uranium that they can, with the development of a chronic type of nephropathy, reestablish to some extent the function of the kidney, while in the older animals these changes of repair do not occur.

As a result of these observations it has seemed advisable to group the experiments used in this study according to the age of the animal. Group 1 is represented by seven animals between 6 and $10\frac{1}{2}$ years of age; Group 2, by eight animals varying from 1 to 4 years of age; while Group 3 includes twelve animals from 5 to 11 months of age. From the experiments in the different groups of animals, the course of the intoxication, as illustrated by one experiment typical for the group, is included in the form of a table. In order to reduce the space required for the tables, only observations on every 3rd day of the experiment have been tabulated.

Group 1. Animals Varying in Age from 6 to $10\frac{1}{2}$ Years.

Seven animals are included in this group. Four of the experiments were artificially terminated, one on the 6th, 8th, 10th, and 12th days respectively following the commencement of the uranium intoxication. Of the remaining three animals of the group two died on the 10th day of the intoxication in a comatose condition, which rapidly developed following a period of convulsions. The third animal died on the 6th day in air-hunger. None of the animals of this group survived the intoxication for longer than 12 days.

Reference to Table I shows the course of the intoxication in an animal representative of this group. During the first 24 hour period following the injection of uranium there occurs an increase

[12] MacNider, W. de B., A consideration of the relative toxicity of uranium nitrate for animals of different ages. I, *J. Exp. Med.*, 1917, xxvi, 1.

in the output of urine. At this early period both albumin and glucose appear in the urine, and the centrifugalized urine shows both hyaline and granular casts. Evidence of kidney injury is indicated by the sudden reduction in the elimination of phenolsulfonephthalein and by a retention of blood urea. In Experiment 1 the output of phenolsulfonephthalein was reduced within 24 hours from the normal elimination of 58 per cent to 20 per cent, while the blood urea showed a retention of 0.028 per cent as opposed to the normal of 0.015 per cent.

The acid-base equilibrium of the blood in this group of old animals has shown a similar abrupt change in favor of a decrease in the alkali reserve. The reserve alkali in Experiment 1 was re-

TABLE I.

Prolonged Uranium Intoxication. Group 1, Experiment 1.

Day of experiment.	Age and weight of animal.	Water in 24 hrs.	Uranium nitrate per kg.	Urine in 24 hrs.	Albumin.	Glucose.	Blood urea	Phthalein.	R. pH.	Carbon dioxide tension.
		cc.	*mg.*	*cc.*	*gm.*	*per cent*	*per cent*	*per cent*		*mm.*
1	7 yrs. 12.35 kg.	500	0	471	0	0	0.015	58	8.1	41
2		500	4	760	1.3	0.9	0.028	20	8.0	37
5		500	0	419	4.0	1.75	0.040	Tr.	7.8	29
8		500	0	58	3.6	0.97	0.078	0	7.75	21

duced from 8.1 to 8, while the tension of alveolar air carbon dioxide was reduced from 41 mm. to 37 mm.

The progress of the intoxication in this group of old animals, as shown by Table I, has consisted in a rapid reduction in the formation of urine, an increase in the amount of both albumin and glucose in the urine, and a progressive decrease in the functional capacity of the kidney, as shown by the elimination of phenolsulfonephthalein and the retention of blood urea. This functional change in the kidney is associated with a rapid decrease in the alkali reserve of the blood and an associated reduction in the tension of carbon dioxide in alveolar air. Both the functional response of the kidney and the reduction in the alkali reserve of the blood are progressive in severity with the duration of the experiment. In

this group of old animals there has been no attempt at a restoration of renal function and no reestablishment of a normal acid-base equilibrium.

The kidneys from the animals of this group have shown no characteristic gross pathological change. The surface is smooth and the capsule has not been adherent. On section the cortex is pale. The tissue at the corticomedullary junction has shown a large amount of fat, which extends into the cortex in streaks. The histological study has shown a degree of degeneration which depends for its severity and extent upon the duration of the intoxication. In the animals of this group in which the kidneys were obtained prior to the 8th day, the degenerative changes have been largely confined to the tubules. These changes have consisted in an acute swelling and vacuolation, which first appears in the third portion of the convoluted tubule. This change is followed by pyknosis of the nuclei and necrosis of the epithelium. Even at this early stage of the intoxication the ascending limb of Henle's loops contains a large amount of fat. The accumulation of fat is more marked in the kidneys of this older group of animals than in any of the remaining younger groups.

By the 10th to the 12th days of the intoxication the epithelial injury has become more diffuse. All the proximal convoluted tubule has participated in the degeneration, and the epithelium of the distal convoluted tubules and junctional tubules has shown evidence of injury. In addition to the extension of the epithelial injury the vascular tissue of the kidney becomes involved in the degeneration. The intertubular and glomerular capillary endothelium has shown a peculiar hyaline type of change, and later becomes vacuolated. Following this degeneration of the vascular tissue exudates occur between the tubules and into the capsular space of the glomeruli (Fig. 1).

In this group of old animals there has been no indication of an attempt to repair the injury. There is no evidence of nuclear division.

Group 2. Animals Varying in Age from 1 to 4 Years.

Eight animals are included in this group. Three of the experiments were terminated on the 6th day and two on the 10th day. Of the remaining animals, one died on the 14th day and two on the 21st day.

The course of the intoxication in the animals of this age grouping resembles in general the response obtained with the older animals of Group 1.

The injection of the animals of this group with 4 mg. of uranium nitrate is followed within 24 hours by the appearance of both albumin and glucose in the urine (Table II). Following an initial increase in the formation of urine the daily output is rapidly reduced so that by

TABLE II.

Prolonged Uranium Intoxication. Group 2, Experiment 2.

Day of experiment.	Age and weight of animal.	Water in 24 hrs.	Uranium nitrate per kg.	Urine in 24 hrs.	Albumin.	Glucose.	Blood urea.	Phthalein.	R. pH.	Carbon dioxide tension.
		cc.	*mg.*	*cc.*	*gm.*	*per cent*	*per cent*	*per cent*		*mm.*
1	3 yrs. 14.7 kg.	500	0	400	0	0	0.015	68	8.0	42
2		500	4	413	0.5	0.61	0.015	63	7.95	37
5		500	0	634	0.7	1.25	0.018	5	7.8	27
8		500	0	450	1.3	1.87	0.023	Tr.	7.8	25
11		500	0	210	0.9	1.03	0.031	"	7.75	21
14		500	0	35	0.5	0.87	0.080	0	7.65	18

the 14th day, as indicated in Experiment 2, only 35 cc. of urine were formed. Within a few hours of the appearance of albumin in the urine there is a reduction in the reserve alkali of the blood, a decrease in the elimination of phenolsulfonephthalein, and a retention of blood urea. These changes in the functional capacity of the kidney and in the acid-base equilibrium of the blood progress in severity with the duration of the experiment.

A study of tables of animals representative of Groups 1 and 2 (Tables I and II) shows the following variations in the response of the animals of the two groups to the intoxication. The older animals of Group 1 show a more rapidly developing intoxication, as indicated by an earlier reduction in the formation of urine, by the urine's contain-

ing a larger amount of both albumin and glucose, and by the more rapid development of a severe kidney injury. In addition to these variations in the severity of the intoxication in the two groups of animals, a similar variation has occurred in the rate with which the alkali reserve of the blood in the two groups shows a depletion. By the 8th day of the intoxication in Experiment 1 of Group 1 the alkali reserve had been reduced to 7.75, while in Experiment 2 of the younger group of animals, Group 2, this degree in the reduction of the alkali reserve was obtained on the 11th day.

A study of the rate with which the kidney shows evidence of injury in the two groups indicates that the kidneys of the older animals are more susceptible to injury than is the case with the younger group of animals. In Experiment 1 of Group 1, within the first 24 hour period the elimination of phenolsulfonephthalein was reduced from 58 per cent to 20 per cent, and a retention of blood urea developed of 0.028 per cent as compared with the normal of 0.015 per cent.

In the younger animals of Group 2, as illustrated by Experiment 2, such a rapid injury to the kidney did not develop. During the first 24 hour period of the intoxication the elimination of phenolsulfonephthalein was only reduced from 68 per cent to 63 per cent, and during this same period there was no retention over the normal in blood urea.

The pathological study of the kidneys from the animals of Group 2 has shown only one outstanding difference from the pathological reaction described as occurring in the animals of Group 1. In both groups the epithelium of the convoluted tubules has shown the most marked evidence of injury. The type of injury has been the same. The kidneys of the younger group of animals, Group 2, that have been obtained after the 6th day of the intoxication, show definite evidence of repair by a beginning regeneration of tubular epithelium (Fig. 2). The regeneration first appears in the third or terminal portion of the convoluted tubule. In the cells of this portion of the tubule which have sufficiently withstood the process of degeneration, new epithelium is formed by a process of indirect cell division. At first the newly formed cells are large and have a clear cytoplasm. Later these cells flatten out and contain a small amount of deeply staining cytoplasm in proportion to the large hypochromatic nuclei.

In Fig. 2 the different stages in the regeneration of atypical cells in the convoluted tubules is clearly shown.

In this second group of animals which differ from the first group in that an attempt at repair has commenced in the kidneys, the processes of repair have not sufficiently progressed to be expressed by any improvement in the functional capacity of the kidney or by any change in the direction of restoring the normal acid-base equilibrium of the blood.

Group 3. Animals Varying in Age from 5 to 11 Months.

Twelve animals are included in this group. Two of the experiments were terminated on the 6th day of the intoxication, four on the 16th day, two on the 20th, two on the 35th day, and the remaining two experiments on the 48th day.

A study of Tables III and IV, of Group 3, shows not only the stage of degeneration in these animals as indicated by the kidney injury and the disturbance in the acid-base equilibrium of the blood, but also the second period in the intoxication, which is characterized by a partial restoration in the functional capacity of the kidney, and by an attempt to restore the normal acid-base equilibrium of the blood.

The response of this youngest group of animals to an intoxication by uranium is similar qualitatively to the response which has been obtained in Groups 1 and 2 of the older animals. The difference in the reaction of these younger animals to uranium is shown by a delay in the development of the kidney injury and by the fact that the alkali reserve of the blood does not show so rapid a depletion as was the case with the older groups of animals. A further study also shows that an animal of this group (Experiment 4, Table IV) may during the course of the intoxication show as marked evidence of kidney injury and as great a depletion in alkali reserve as did the older animals of Groups 1 and 2, yet following this degree of injury the younger animals (Group 3) are able to some extent to return to the normal, while with the older animals such a reparative reaction has not occurred.

In Experiments 3 and 4 (Tables III and IV) these younger animals have shown less albumin in the urine than has been the case with the older animals. During the first 24 hour period of the intoxication

these animals have shown a reduction in the elimination of phenolsulfonephthalein and a retention of blood urea. In these animals, however, the reduction in the elimination of the dye is not so rapid and the retention of blood urea is not so great as is the case with the older groups of animals.

In this group of young animals there is the usual association between the kidney injury and the reduction in the alkali reserve of the blood. This disturbance in the acid-base equilibrium of the blood shows a gradual development. In Experiment 4 (Table IV)

TABLE III.

Prolonged Uranium Intoxication. Group 3, Experiment 3.

Day of experiment.	Age and weight of animal.	Water in 24 hrs.	Uranium nitrate per kg.	Urine in 24 hrs.	Albumin.	Glucose.	Blood urea.	Phthalein.	R. pH.	Carbon dioxide tension.
		cc.	*mg.*	*cc.*	*gm.*	*per cent*	*per cent*	*per cent*		*mm.*
1	8 mos. 15.9 kg.	500	0	418	0	0	0.012	58	8.1	40
2		500	4	623	0.3	0.7	0.016	33	8.05	40
5		500	0	310	1.0	1.19	0.024	22	8.0	40
8		500	0	105	0.6	0.52	0.048	18	7.9	32
11		500	0	230	0.2	0.3	0.070	10	7.9	31
14		500	0	120	0.2	0.1	0.100	0	7.9	32
17	8 mos. 12.3 kg.	500	0	503	Tr.	0	0.200	0	7.9	34
20		500	0	700	0	0	0.206	Tr.	7.9	30
23		500	0	700	0	0	0.208	"	7.9	30
26		500	0	794	0	0	0.218	4	7.95	36
29		500	0	628	0	0	0.180	8	7.95	38
32		500	0	623	Tr.	0	0.188	10	7.95	38
35		500	0	629	0	0	0.163	10	8.0	38

the greatest reduction in alkali reserve was 7.75, which was obtained on the 12th day of the experiment. In Experiment 3 the greatest reduction was 7.9. This reading was first obtained on the 8th day of the intoxication.

Judging by the percentage elimination of phenolsulfonephthalein, the degree of retention of blood urea, and the maximum reduction in the alkali reserve of the blood, we find that the height of the intoxication by uranium in these animals is reached between the 8th and 15th days of the experiment. Following this period, in this group of young

animals, the functional capacity of the kidney shows a beginning improvement and the alkali reserve of the blood shows less depletion. The histological study of the kidney during such a period of functional recuperation shows, in addition to the evidence of repair in the connective tissue element, a progressive epithelial regeneration.

In Experiment 4, Group 3, (Table IV), the height of the intoxication was reached on the 15th day of the experiment. At this stage

TABLE IV.

Prolonged Uranium Intoxication. Group 3, Experiment 4.

Day of experiment.	Age and weight of animal.	Water in 24 hrs.	Uranium nitrate per kg.	Urine in 24 hrs.	Albumin.	Glucose.	Blood urea.	Phthalein.	R.pH.	Carbon dioxide tension.
		cc.	*mg.*	*cc.*	*gm.*	*per cent*	*per cent*	*per cent*		*mm.*
1	11 mos. 12.58 kg.	500	0	605	0	0	0.016	81	8.0	42
2		500	4	619	0.75	0.81	0.028	56	7 95	38
3		500	0	211	1.2	0.85	0.038	51	7.9	34
6		500	0	189	0.4	0.27	0.077	31	7.9	34
9		500	0	475	Tr.	Tr.	0.177	8	7.85	31
12		500	0	192	0	"	0.180	Tr.	7.75	20
15		500	0	397	0	0	0.297	0	7.75	24
18		500	0	653	Tr.	0	0.183	24	7.8	27
21		500	0	611	"	0	0.108	24	7.8	27
24	11 mos. 9.5 kg.	500	0	459	Tr.	0	0.073	29	7.8	27
27		500	0	561	"	0	0.082	35	7.85	30
30		500	0	355	0.25	0	0.045	37	7.95	37
33		500	0	283	0.6	0	0.120	21	7.9	36
36		500	0	523	0.7	0.34	0.054	21	7.95	36
39		500	0	525	0.2	0	0.061	16	8.0	40
42		500	0	610	0.2	0	0.082	21	8.0	40
45		500	0	531	Tr.	0	0.053	28	8.0	40
48		500	0	566	"	0	0.065	28	8.0	40

of the experiment the animal gave a negative phenolsulfonephthalein test, the blood urea showed a retention of 0.297 per cent, and the alkali reserve of the blood had been reduced to 7.75. On the 18th day the animal had an elimination of phenolsulfonephthalein of 24 per cent, a reduction in the retention of blood urea to 0.183 per cent, and an increase in the alkali reserve of the blood to 7.8. During the further course of the experiment, which was terminated on

the 48th day, there occurred a gradual increase in the elimination of phenolsulfonephthalein, a decrease in the retention of blood urea, and an increase in the alkali reserve of the blood until the 30th day of the intoxication. At this period there occurred an unexplained relapse in the animal's condition. The phenolsulfonephthalein elimination dropped to 21 per cent and during the following 6 days went as low as 16 per cent. With this change in the ability of the kidney to eliminate the dye, the blood urea rose from a retention of 0.045 per cent to a retention of 0.120 per cent. The alkali reserve of the blood was reduced from 7.95 to 7.9. Following this period of secondary injury another phase of improvement developed, so that at the termination of the experiment on the 48th day the animal had an elimination of phenolsulfonephthalein of 28 per cent, a blood urea of 0.065 per cent, and a reserve alkali reading of 8.0.

The second experiment representative of this group of young animals (Experiment 3, Table III) shows a type of response similar to that of Experiment 4, which has been discussed in detail. Experiment 3 was terminated on the 35th day of the intoxication, 6 days after the functional response of the kidneys gave evidence of an improvement in the degree of intoxication and after the beginning restoration of the acid-base equilibrium of the blood.

The pathology of the kidney in the experiments of Group 3 has been studied at two periods of the intoxication. Experiments have been terminated and the kidneys obtained for study within 10 days after the functional observations showed the period of improvement in the animals to have been established. In other animals of the group the experiments were terminated after the stage of improvement had persisted for a longer period, 20 to 38 days. By such a selection of tissue not only have observations been made of the processes of repair in the kidney, but it has been possible to study these changes in connection with the reestablishment of the functional capacity of the kidney.

The kidneys obtained from animals in the early stages of improvement from the intoxication show no gross evidence of disease.

The histological study shows a late stage in the regeneration of tubular epithelium (Fig. 3). The regenerated epithelium remains of the flattened atypical type. The cytoplasm which stains deeply has

differentiated into cell units. The nuclei are hyperchromatic and of large size in proportion to the surrounding cytoplasm. Most of the granular detritus, the result of the early epithelial necrosis, has been removed from the newly lined tubules. In some of the tubules regeneration has failed to develop. Such tubules are filled with necrotic material. A diffuse formation of intertubular connective tissue has occurred which is in the cellular stage.

The vascular pathology has consisted in an invasion of the glomeruli by fibroblasts with a matting together of the capillary loops. The capsules of the glomeruli have shown an early thickening with a marked periglomerular cellular fibrosis (Fig. 4).

The kidneys obtained from animals late in the period of recovery from the intoxication have failed to show any gross change, such as an adherent capsule, scarring of the cortex, or any marked decrease in the proportionate relation between the cortex and medulla.

The histological study of this tissue has shown a later stage in the process of repair than was shown by tissue obtained from animals soon after a beginning recovery from the intoxication had been established. The sections have shown an increase in the number of tubules containing flattened, regenerated epithelium. Tubules with degenerating and necrotic epithelium are less numerous. The glomeruli show a later stage of fibrosis and the capsules show newly formed connective tissue fibers. The periglomerular and intertubular connective tissue has passed from the cellular stage to the stage of laying down connective tissue fibers (Fig. 5).

DISCUSSION.

The experiments conducted in this investigation confirm the earlier work of Dickson, who demonstrated that uranium would produce in certain of the lower animals a chronic kidney injury comparable with certain of the chronic diffuse nephropathies of man. The experiments furthermore demonstrate the character and the severity of the functional disturbance associated with the various stages of the uranium intoxication. In these experiments it has been shown that the severity of the acute degenerative changes in the kidney is largely dependent upon the age of the animal. The older

animals develop a more rapid and a severer type of intoxication than is the case with the younger animals. The intoxication is characterized by a reduction in the alkali reserve of the blood and by the development of a kidney injury. The injury to the kidney is expressed functionally by the appearance of albumin in the urine, a reduction in the elimination of phenolsulfonephthalein, and by a retention of blood urea. The reduction in the elimination of the dye occurs as the first indication of renal injury. The reduction progressively increases with the severity of the degeneration in the kidney. The retention of blood urea is a somewhat later manifestation of kidney injury. A retention of blood urea may be deferred for 6 to 36 hours after the development of a marked reduction in the ability of the kidney to eliminate phenolsulfonephthalein. Subsequently, during the period of acute degeneration, the retention of blood urea shows a progressive increase.

Albumin has appeared in the urine of all the animals during the first 24 hours. Following its appearance the amount of albumin increases for the first 2 to 6 days of the experiment, and during this period the quantitative output of albumin has shown a relation with the renal functional tests. After this period, however, the amount of albumin in the urine rapidly decreases, while the reduction in the elimination of phenolsulfonephthalein and the retention of blood urea progressively increase. From this observation it would appear that quantitative determinations of the amount of albumin in the urine may give very imperfect information concerning the degree of renal injury.

All the animals intoxicated by uranium have shown a disturbance in the acid-base equilibrium of the blood, as indicated by a reduction in the alkali reserve and by a decrease in the tension of alveolar air carbon dioxide. The depletion in the alkali reserve has developed more rapidly and has shown a greater degree of reduction early in the experiments on the older animals than has been the case with the younger animals. The severity of the intoxication, as expressed by the degree of functional disturbance of the kidneys, has shown a parallel with the severity of the disturbance in the acid-base equilibrium of the blood.

A study of the intoxication in the animals of the various age groups shows that the older animals develop a type of kidney injury in which there is no attempt at regeneration, and therefore no reestablishment of renal function. In these animals there has failed to develop any attempt to restore the normal acid-base equilibrium of the blood. In the younger animals, however, epithelial regeneration does occur, and following this attempt at repair the functional capacity of the kidney improves and the depletion of the alkali reserve of the blood is in part restored. The earliest evidence of such functional restoration has consisted in a return of the ability of the kidney to eliminate phenolsulfonephthalein. Following this improvement the percentage retention of blood urea is decreased and still later the acid-base equilibrium of the blood returns towards the normal. In two of the younger animals during the period of recovery from the intoxication, the acid-base equilibrium of the blood was restored to the point of normality, yet in these animals the retention of blood urea remained high and the elimination of phenolsulfonephthalein was sufficiently low to indicate the existence of a severe grade of kidney injury.

CONCLUSIONS.

1. Uranium nitrate is relatively more toxic for old animals than for young animals.
2. This relative toxicity is not only expressed in the old animals by a greater functional disturbance of the kidney, but is also shown by an inability on the part of these animals to repair the kidney injury and reestablish its functional capacity.
3. The intoxication in younger animals has been followed by a repair of the renal injury and a partial restoration of kidney function.
4. In these animals the processes of repair lead to the development of a chronic diffuse type of nephropathy in which the acid-base equilibrium of the blood may be maintained at the point of normality. In these animals renal functional tests indicate the presence of severe kidney injury.

EXPLANATION OF PLATES.

Plate 26.

Fig. 1. Camera lucida drawing, Leitz oc. 2, obj. 6. The figure is from the kidney of the animal of Experiment 1, Group 1 (Table I). It shows at *A* an acute swelling and vacuolation of the convoluted tubule epithelium. At *B* are shown tubules in which the epithelial degeneration has advanced to the stage of complete necrosis. At *C* is shown a junctional tubule distended with necrotic epithelial debris. Between the convoluted tubules at *D* there has occurred an extravasation of blood.

Plate 27.

Fig. 2. Camera lucida drawing, Leitz oc. 2, obj. $\frac{1}{12}$. Oil immersion. The figure is from the kidney of the animal of Experiment 2, Group 2 (Table II). It represents the different stages in the regeneration of a flattened atypical type of convoluted tubule epithelium. At *A* is shown a regenerated epithelial cell containing a mitotic figure. This tubule is partially lined by regenerated cells. The tubule also contains the remains of necrotic cell material. At *B* and *C* are shown tubules in the early stage of epithelial regeneration. At *D* is shown a tubule in a later stage of the process of epithelial regeneration. The large epithelial cells which are first regenerated are here flattening out to line the tubule with an atypical and less specialized type of epithelium, which is characterized by large hyperchromatic nuclei and a small amount of deeply staining cytoplasm.

Plate 28.

Fig. 3. Camera lucida drawing, Leitz oc. 2, obj. 6. The figure is from the kidney of the animal of Experiment 3, Group 3 (Table III). It shows a later stage in the regeneration of tubular epithelium and the stage in the delayed uranium intoxication which is characterized by the formation of intertubular connective tissue. At *A* are shown convoluted tubules lined by a flattened type of epithelium. The cytoplasm has differentiated with the formation of definite cell boundaries. The necrotic material has been removed from the lumen of the tubules. At *B* are shown tubules filled with the remains of necrotic epithelium. No epithelial regeneration has developed in these tubules. At *C* is shown the early formation of cellular connective tissue.

Plate 29.

Fig. 4. Camera lucida drawing, Leitz oc. 2, obj. 6. This figure is taken from the kidney of the same animal as Fig. 3. It shows at *A* a glomerulus in an early stage of connective tissue infiltration. Connective tissue nuclei are numerous. The capillary loops have become obliterated and matted together. At *B* is shown an early thickening of the capsule of the glomerulus and a marked peri-

glomerular cellular fibrosis. At *C* are shown tubules lined with regenerated flattened epithelium. At *D* convoluted tubules may be seen in an early stage of degeneration. At *E* is shown a marked intertubular cellular fibrosis in which are several newly lined tubules.

PLATE 30.

FIG. 5. Camera lucida drawing, Leitz oc. 2, obj. 6. The figure is from the kidney of the animal of Experiment 4, Group 3 (Table IV). It shows an advanced stage of repair following the uranium intoxication which has resulted in the development of a chronic diffuse type of nephropathy. At *A* is shown the predominance of regenerated tubules, lined by flattened epithelium. At *B* is shown a shrunken glomerulus with a capsule thickened by the laying down of connective tissue fibers. At *C* the intertubular connective tissue has passed from the early cellular stage into the stage of connective tissue fiber formation.

Fig. 1.

(MacNider: Chronic nephropathy by uranium nitrate.)

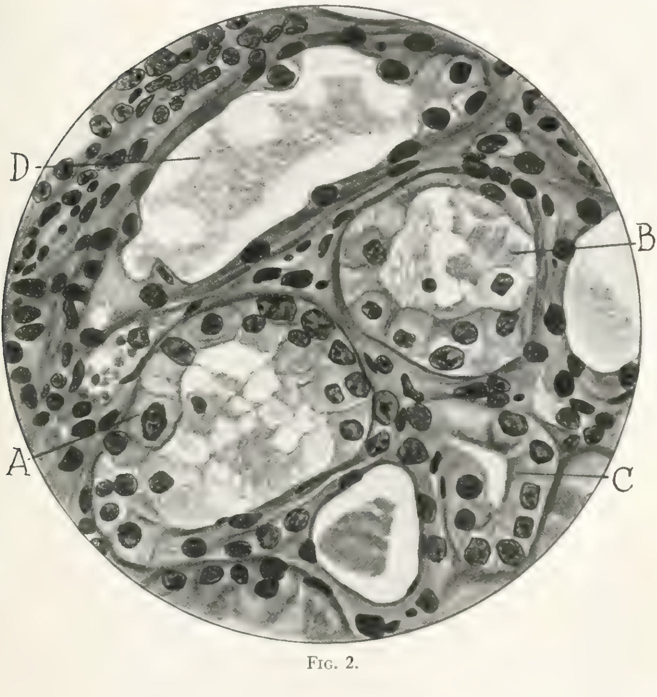

Fig. 2.

(MacNider: Chronic nephropathy by uranium nitrate.)

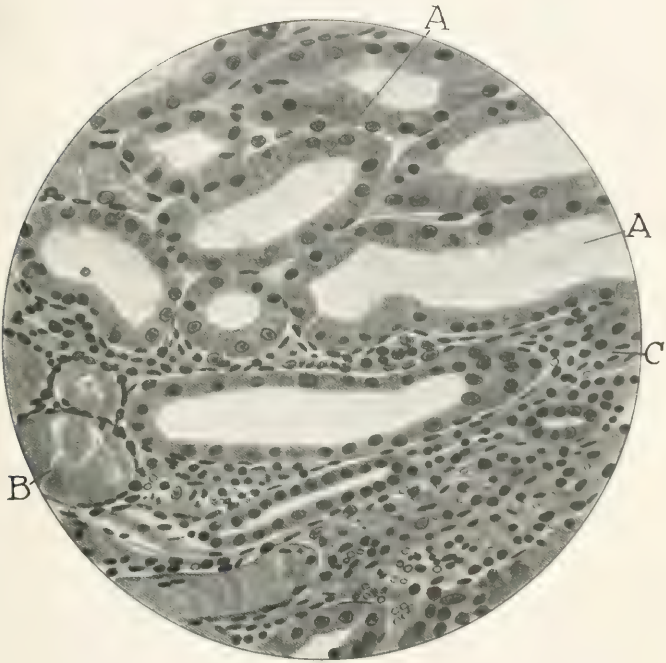

Fig. 3.

MacNider. (Chronic nephropathy by uranium nitrate.)

FIG. 4.

(MacNider: Chronic nephropathy by uranium nitrate.)

Fig. 5.

(MacNider: Chronic nephropathy by uranium nitrate.)

[Reprinted from The Journal of the American Medical Association, May 3, 1919, Vol. lxxii, No. 18, p. 1289.]

A TEST FOR GLOBULIN IN SPINAL FLUID FOR USE AT THE BEDSIDE.

By HAROLD L. AMOSS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Rough tests on globulin solutions showed the isoelectric point to be between p_H 4.4 and 4.7. The p_H value for a 2 per cent. solution of anhydrous potassium dihydrogen phosphate is 4.4. If the globulin precipitation depends on the p_H value of the solution, then a solution of primary phosphate could be substituted for the Noguchi butyric acid sodium butyrate mixture. Various concentrations of the primary phosphate were used, from 2 per cent. to 10 per cent., in testing spinal fluids known to contain excess of globulin, as shown by the Noguchi method. To 0.2 c.c. of the spinal fluid were added 0.6 c.c. of the primary phosphate solution, and the tube containing the mixture was immersed in boiling water for six minutes. The solution acting the most quickly and yielding the largest flocculation was the 3 per cent.; this compares favorably with the Noguchi test.

Change in p_H Value in Boiling.

The p_H values of the mixtures of spinal fluid plus phosphate solution and the mixtures of spinal fluid plus Noguchi's mixture are equal. However, on boiling, the phosphate spinal fluid mixture becomes less acid, as shown by methyl red, while the Noguchi mixture remains unchanged. If the phosphate solution is fortified by the addition of acetic acid so that the p_H value after boiling with the spinal fluid remains about 4.4, the globulin is either not precipitated or the floccules are very minute. Apparently other factors than hydrogen ion concentration enter into the reaction. A 10 per cent. phosphate solution possesses sufficient buffer power to maintain the p_H value during boiling, but the high salt content produces very fine granulation of the globulin. Addition of various amounts of sodium chlorid yields like results. It appears that the globulin precipitation depends not only

on the hydrogen ion concentration but also on the amount of salts present.

Spinal fluids tend to become more alkaline even when placed in the ice box over night. For testing such fluids it has been found advisable to add to each 100 c.c. of potassium dihydrogen phosphate solution 0.05 c.c. of glacial acetic acid.

Comparison of the Phosphate Solution Reagent with the Noguchi Test.

Through the kindness of Dr. Martha Wollstein of The Rockefeller Institute and Miss Innerstein of the New York City Board of Health Laboratory, twenty-one specimens of spinal fluids were tested with the Noguchi and the 3 per cent. primary phosphate-acetic acid solution. Two-tenths c.c. of the spinal fluid was used in each test. In the Noguchi test 0.5 c.c. of 10 per cent. butyric acid in isotonic sodium chlorid solution was added and the mixture heated for two minutes. One-tenth c.c. of normal potassium hydroxid was then added and the tube containing the mixture placed in boiling water for four minutes. The phosphate-acetic acid mixture test was conducted as follows: To 0.2 c.c. of the spinal fluid there was added 0.6 c.c. of the reagent. The tube containing the mixture was placed in boiling water for six minutes. The results of the test are shown in the accompanying table.

The reagent, being less delicate than that of the Noguchi test, gives no precipitate with normal fluids, but consistently indicates fluids in which globulin is present in excess. The reaction requires about two minutes longer heating than is required with the Noguchi test, but requires less manipulation, since one solution is used instead of two. Accurate pipetting of the spinal fluid only is necessary; slight variations in the amount of the reagent added do not affect the end-result. The objectionable odor of the butyric acid is absent; consequently the test can be made in the patient's room. Should small amounts of the solution be spilt in transportation, the diagnostician's kit is not thereby rendered disagreeably redolent. The solution is quickly and easily prepared and is stable, while the Noguchi reagents require accurate titration and tend to become cloudy on standing.

Comparison of Results of Globulin Tests on Spinal Fluids with the Noguchi Reagents, and with 3 Per Cent. Potassium Dihydrogen Phosphate Acetic Acid.

Spinal Fluid from Case of:	Butyric Acid Test of Noguchi	Solution:—3 Per Cent. KH_2PO_4 and 0.05 Per Cent. Glacial Acetic Acid
1. Encephalitis lethargica (?)	+	+*
2. Encephalitis lethargica	±	–
3. Tuberculous meningitis	++++	++++
4. Normal fluid	±	–
5. Cerebrospinal meningitis	+++	+++
6. Tuberculous meningitis	++++	++++
7. Cerebrospinal meningitis	++++	++++
8. Cerebrospinal meningitis	++++	++++
9. Normal fluid	±	–
10. Tuberculous meningitis	++++	++++
11. Normal fluid	±	–
12. Encephalitis lethargica (?)	++	++*
13. Normal fluid	+	– slight turbidity
14. Cerebrospinal meningitis	++++	++++
15. Encephalitis lethargica (?)	++	++*
16. Cerebrospinal meningitis	++++	++++
17. Encephalitis lethargica (?)	+	+*
18. Encephalitis lethargica	+	±
19. Encephalitis lethargica	+	+*
20. Encephalitis lethargica	++	+*
21. Encephalitis lethargica	+	+*

* Floccules smaller than in Noguchi test.

SUMMARY.

A suitable reagent for the globulin test in spinal fluid may be prepared by dissolving 3 gm. of anhydrous potassium dihydrogen phosphate in 100 c.c. of distilled water and adding 0.05 c.c. of glacial acetic acid. In making the test, 0.2 c.c. of the spinal fluid plus 0.6 c.c. of the reagent are mixed in a small agglutination tube and placed in boiling water for six minutes. This test is slightly less delicate than the Noguchi test, but offers some advantages over the latter for field work.

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THE BLOOD-PRESSURE CURVE FOLLOWING AN INTRA-SPINAL INJECTION OF ADRENALIN.

BY J. AUER AND S. J. MELTZER.

From the Department of Physiology and Pharmacology of The Rockefeller Institute for Medical Research.)

(Received for publication, September 30, 1918.)

The discovery by Oliver and Schäfer that adrenal extract causes a rise of blood pressure was made by administering the extract intravenously. The blood-pressure curve which follows such an injection is characteristic. Practically without a latent period, the pressure curve rises and reaches its maximum height in a very short time. There is almost no plateau; the descent is gradual; the original pressure is attained in about five to seven minutes. Sometimes the pressure falls for a short period below the original level.

We studied at various times the character of the pressure curve which follows after diverse methods of administration of adrenalin. Among others, we studied the pressure curve which follows intrapinal injection of adrenalin in monkeys under the generally accepted supposition that absorption from the spinal canal is a slow process. The results were quite interesting. In most instances the blood-pressure curve was characteistic; the latent period was prolonged, the rise was comparatively slow, the maximum, although quite high, was only attained after several minutes and continued plateau-like for a fairly long time; the descent to normal was, comparatively speaking, very slow. These findings were embodied in a brief communication before the Society for Experimental Biology and Medicine (1).

About simultaneously with our report there appeared a brief communication of Dixon and Halliburton on the rapidity of the absorption of drugs into the cerebro-spinal fluid (2). There they stated that:

> A small dose of adrenalin (1 cc. of 1 in 10,000) solution, will equally rapidly produce a typical rise of arterial blood pressure, in fact almost as high as if it were injected intravenously.

In their experiments the drug was introduced through a cannula inserted into the subcerebellar cisterna. In a later article however, Dixon and Halliburton (3) make the following statement:

> Since the publication of our preliminary note, Auer and Meltzer have made some intraspinal injections of adrenalin which they expected would have no stronger action on blood pressure than that of a subcutaneous injection. They employed monkeys and injected large doses; the arterial pressure rose slowly and reached its maximum in a few minutes and remained high for a considerable time. The injections were made into the lumbar region. We have made some injections into the lumbar region but there, as we have already stated, absorption is comparatively slow and several minutes are required before any noticeable effect is observed.

Thus there is no contradiction between our observations and the results obtained by Dixon and Halliburton. In the last mentioned communication they rather confirmed our observations that when the injection of adrenalin is given in the lumbar region the absorption is comparatively slow, and several minutes are required before any noticeable effect is observed.

Dixon and Halliburton, however, fail to make any statement regarding the final intensity or the long duration of the effect. These authors were apparently interested mostly in that part of our communication which seems to contradict their statement, namely, that the absorption of adrenalin from the cerebro-spinal fluid is almost as rapid as if it were injected intravenously and confined themselves to the explanation of the seeming contradiction by the fact that our experiments were made by injections into the lumbar region while they made their injections into the subcerebellar cisterna. They were not interested in the details of the effect of intralumbar injections.

To us it seems that the most interesting part of our observations consists in the facts that the maximum of the blood-pressure rise following an intraspinal injection of adrenalin has a comparatively long plateau and the descent of the curve to the normal level is very slow; in other words, that the blood-pressure rise following an intraspinal injection of adrenalin is as a rule the longest ever observed after a single injection of adrenalin. This fact is interesting from a theoretical point of view; but it may, as we have pointed out elsewhere (4) have an important practical application. We intend therefore to describe

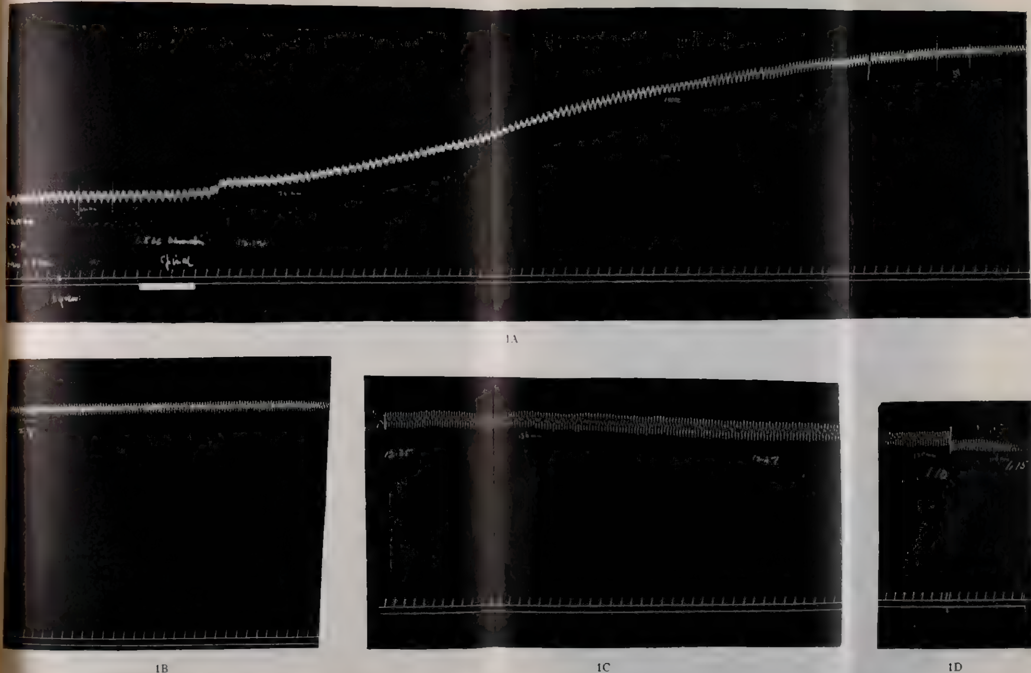

FIG. 1 The four parts of figure 1 belong to a blood-pressure curve obtained from an *intraspinal* injection in the lumbar region of 1.5 cc. adrenalin in a monkey. On all the figures the upper line shows the blood pressure, the middle line marks the time in four seconds and is also the base line of the blood-pressure curve. On figure 1A the stimulation line carries a mark indicating the time and duration of the injection of adrenalin. Before the injection the blood pressure was 62 mm. After the injection the blood pressure began to rise slowly; after 30 seconds the blood pressure was only 74 mm.; after 3 minutes it was 140 mm. Figure 1B (continuation of figure 1A) after 5 minutes blood pressure was 170 mm., about 7 minutes after the injection the blood pressure was 172 mm. This was the maximum, which continued for several minutes with practically very little change (plateau-like). Then it began gradually to fall. Figure 1C (the continuation of 1B) shows that about 24 minutes after the injection the pressure was still 138 mm. and the decline was going on very slowly. Figure 1D shows that at 1.10, that is, 58 minutes after the injection, the blood pressure was 120 mm. and even at 1.15, that is, 1 hour and 3 minutes after injection, the blood pressure was still 114 mm. mercury. These figures show then that 1 hour and 3 minutes after an intraspinal injection of adrenalin the pressure was still high above the original blood pressure—62 mm. at 12.12 p.m. and 114 mm. at 1.15 p.m.

the results of our experiments in some detail and, especially, to reproduce a most striking curve to illustrate the effects of an intraspinal injection of adrenalin. The present high price of monkeys prohibits a repetition of our experiments on a large scale, but through the courtesy of Doctor Flexner we were enabled some time ago to make a few observations on three monkeys. These observations will be included in the following general statement.

EXPERIMENTAL.

Except in monkeys and human beings, it is impossible in other animals for an intraspinal injection in the lumbar region to enter the subarachnoidean space of the canal below the spinal cord; in most cases the needle enters into the cord itself and the injection is made into its tissue. In monkeys an injection below the spinal cord is frequently possible when the needle is inserted between the third and fourth or between the fourth and fifth lumbar vertebrae. However, even in monkeys the injection is not successful in every case. We have learned in the experiments with adrenalin as well as in our extensive experience with magnesium sulphate that when the escape of spinal fluid through the needle is very scant, the effect of an injection is frequently poor and not characteristic. Furthermore, on account of a venous plexus on the ventral surface of the canal and the minuteness of the cavity of the subarachnoidean space below the cord, the hypodermic needle sometimes enters into the venous plexus and the result of the injection is either that of a mere intravenous injection or, more frequently, of a mixture of an intravenous and an intraspinal injection; in the latter case the injected quantity is too small to produce a characteristic effect of an intravenous as well as of an intraspinal administration. In human beings the condition is apparently much more favorable. In the numerous injections of adrenalin made intravenously in children in the course of the epidemic of poliomyelitis (5) the effect of adrenalin upon the region of the cervical cord was unmistakable whether the quantity injected amounted only to 1 cc. or to 6 cc. for the small children afflicted with the disease.

Our experiments with the lumbar injection of adrenalin were made on ten small monkeys (macacus rhesus). The injections were as a

rule made in the spaces between the third and fourth or fourth and fifth lumbar vertebrae. In a few instances the injection was given in the dorsal region with an effect generally characteristic for injections in the lumbar region. Each animal was used for several experiments after intervals of hours or several days. The smallest dose employed was 0.5 cc. and the largest 1.5 cc. of a 1: 1000 solution of adrenalin.

The character of figure 1 reproduced in this paper is typical for most of our results although we have to state that it represents the most striking result which we have obtained in our series of experiments. It was obtained January 17, 1912, from a second injection of 1.5 cc. adrenalin into the lumbar region of a male monkey; no spinal fluid was obtained at this (second) lumbar puncture. The original blood pressure was 62 mm. mercury. There was no initial drop of blood pressure following the injection, and the rise began after a latent period of eight seconds. The maximum height of 172 mm. was reached in six minutes. The maximum level presented practically a plateau of several minutes duration. The pressure fell exceedingly slowly; for one hour after the injection the blood pressure was still 48 mm. of mercury above the original level (see figs. 1 A, 1 B, 1 C and 1 D).

Figure 2 is from an intravenous injection of 1 cc. of adrenalin administered to a male monkey (2260 grams) November 8, 1917. This tracing is reproduced here for the purpose of emphasizing the contrast between intraspinal and intravenous injections of adrenalin. In this experiment the original pressure was 40 mm. mercury. There was no drop or obvious latent period preceding the beginning of the blood-pressure rise; in eight seconds the maximum of 148 mm. mercury was reached; there was no plateau. After eight minutes the blood pressure was again 47 mm. mercury, a level only slightly higher than the original blood pressure.

Without entering upon the details of our experiments, we may make the following general statement. With negligible exceptions, we may say that the blood-pressure elevation brought about by an intraspinal injection is characterized by a slow rise, by a "plateau-like" perseverance of the maximum rise, by a comparatively very slow fall to the normal level and, in general, by a long duration of the pressor effect when compared with an intravenous injection. These blood-pressure

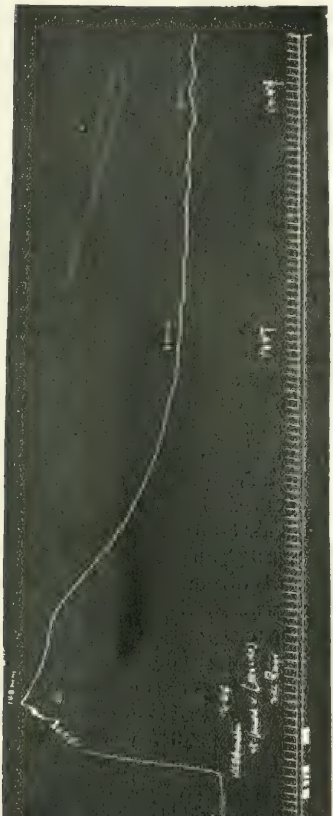

Fig. 2. Shows the blood-pressure curve after an *intravenous* injection of 1 cc. adrenalin into a monkey. The first line represents the pressure curve (pulse pressure very small), the second carries the time markings (4 seconds) and marks the base line while the third line shows the time of the injection of adrenalin and the washing down of adrenalin by 2 cc. of a Ringer solution. The blood pressure before the injection was only 40 mm. The injection of 1 cc. of adrenalin into the right femoral vein raised the blood pressure abruptly and the immediate injection of 2 cc. of Ringer solution, to wash out the adrenalin remaining in the cannula, brought the blood pressure to its maximum height of 148 mm.; the maximum was thus attained 20 seconds after the injection of adrenalin. About 5 minutes later the pressure fell to 64 mm. and gradually came down to a level which in this case remained slightly above the original low blood pressure.

curves vary with the individual animal and its health; with the presence of fluid in the spinal cavity below the cord; with the quantity of adrenalin injected and to a degree also with the number of injections of adrenalin administered to the same animal and the length of the interval between the injections, and especially with the place of injection, for a lumbar injection is distinctly more effective than a thoracic one.

In some instances the very first effect of an intraspinal injection was an initial drop in blood pressure. The drop was usually of short duration and was followed practically in all cases by a characteristic rise. In one case after a lumbar injection of 1.5 cc. the drop was as great as 52 mm. mercury, the pressure falling from 116 to 64 mm. but it soon began to rise and in four minutes reached a height of 162 mm. mercury. In one animal however, after an injection of 1 cc. (no spinal fluid) the blood pressure slowly fell 10 mm. with no subsequent rise of pressure. This monkey was sick. However, after a second injection of 1 cc. adrenalin into the same monkey, no drop of blood pressure occurred and after a latent interval of forty seconds the pressure rose in eight minutes from 100 mm. to 138 mm.; this rise lasted twenty-two minutes. In these two injections the puncture yielded no spinal fluid. Later, in the same animal, after an injection of 1.5 cc. adrenalin, there was first a short preliminary rise followed by a drop to 86 mm. mercury lasting twenty-four seconds and then, during two minutes, the blood pressure rose to a maximum of 143 mm.; after forty-seven minutes the pressure sank again to 84 mm. In this instance the puncture had shown the presence of spinal fluid. In another animal an injection of 1 cc. caused a drop from 72 to 48 mm., that is a drop in blood pressure of 24 mm. mercury which was not followed by any rise. However, twenty-eight minutes later, after a second injection of 1 cc. there was neither a drop of blood pressure nor was there any latent period, but in the course of three minutes after this injection the blood pressure rose from 50 mm. to 124 mm. of mercury. (Study of the further course of blood pressure in this experiment was prevented by the formation of arterial clots.)

The preceding results were observed after injections into the lumbar region. We have, however, records of a few observations on the effects of intraspinal injections when the site of application was the

thoracic region of the cord. In one instance, after an injection of 1.5 cc. of adrenalin into the fifth thoracic interspace, there was no initial drop of blood pressure; a latent period of thirty-six seconds elapsed before the rise began and in two minutes the pressure rose from 54 mm. mercury to 196 mm.; after twenty-four minutes the pressure was still 100 mm. of mercury. In another animal an injection of 1 cc. of adrenalin in the fifth thoracic vertebral interspace brought an initial drop of blood pressure of 25 mm. which lasted two minutes. Then in the course of the following five minutes the blood pressure rose from 102 mm. to a maximum of 170 mm. and after fifteen minutes the blood pressure was still 134 mm. mercury. In general, however, the rise of blood pressure after intrathoracic injections of adrenalin was neither very high nor of striking duration.

CONCLUSIONS.

Intraspinal injections of 1 cc. or 1.5 cc. of adrenalin in the lumbar region of monkeys cause a rise of blood pressure distinctly different in character from the curve obtained after the intravenous injection of adrenalin. The curve of blood pressure following an intraspinal injection is generally characterized by a slow rise from the original level to the maximum height; by a plateau-like duration of the maximum; by a slow fall to the blood pressure which prevailed before the injection of adrenalin. It may be stated in general that an intralumbar injection of adrenalin in monkeys causes a much more lasting effect upon the rise of blood pressure than an intravenous injection. It may perhaps be added that, as a rule, after intraspinal injections the pressure at the end of the pressor effect has not been observed to fall below the original level.

The question discussed in this paper has not been studied in human beings. While there is sufficient experience to show that an intralumbar injection of adrenalin in human beings is apparently innocuous, the effect of such an injection upon the blood pressure has not been studied properly. When we consider, as we stated before, that in human beings an intralumbar injection can be carried out with greater safety and certainty than in monkeys, the advisability of such studies in clinical physiology is quite evident.

BIBLIOGRAPHY.

(1) Auer and Meltzer: Proc. Soc. Exper. Biol. and Med., 1912, ix, 79.
(2) Dixon and Halliburton: Journ. Physiol., 1912, xliv, p. vii.
(3) Dixon and Halliburton: Journ. Physiol., 1916, l, 198.
(4) Auer and Meltzer: Journ. Amer. Med. Assoc., 1918, lxx, 70.
(5) See W. L. Carr's paper on Poliomyelitis read at the Amer. Ped. Soc. (Arch. Ped., 1917, August); also Haas: Med. Rec., 1916, xc, 425.

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ON CERTAIN AROMATIC AMINES AND CHLOROACETYL DERIVATIVES.

BY WALTER A. JACOBS, MICHAEL HEIDELBERGER, AND IDA P. ROLF.
(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received December 31, 1918.)

The compounds described in the present paper represent intermediates in the preparation of a number of aromatic arsenic compounds which are included in a series of papers now being prepared for publication. It is desired to record these intermediates separately in order not to encumber the final papers with descriptions of non-arsenical compounds. It is also hoped that many of these substances, as well as the methods employed in their preparation, may prove of sufficient intrinsic interest to justify the present paper on general grounds.

EXPERIMENTAL.

o-Chloroacetylaminophenol.—This substance was first obtained by Aschan[1] and later by the present authors.[2] More recent experiments have shown the following method to be most suitable for the preparation of this compound: 22 g. *o*-aminophenol are dissolved in 250 cc. of dry acetone, the solution is then chilled, and treated drop by drop, with stirring, with 8.5 cc. (1 mol.) chloroacetyl chloride. 75 cc. 2 *N* aqueous sodium hydroxide are then added in one portion and the mixture further treated with 8.5 cc. chloroacetyl chloride. After acidifying to congo-red with hydrochloric acid the mixture is concentrated to dryness *in vacuo* and the residue suspended in water, filtered off, washed with water, and recrystallized from 50% alcohol. The yield was 31 g., melting at 138–40°.

[1] *Ber.*, **20**, 1524 (1887).
[2] *J. Biol. Chem.*, **21**, 131 (1915).

o-Chloroacetylaminophenyl Acetate.—On adding a drop of concentrated sulfuric acid to a mixture of 2.5 g. *o*-chloroacetylaminophenol and 5 cc. acetic anhydride a clear solution was at once obtained. After warming on the water bath for ten minutes the solution was cooled, treated with water, and the resulting crystals recrystallized from 85% alcohol and benzene. The melting point was then constant at 113.5–4.5°, with preliminary softening. The acetate is sparingly soluble in the cold in acetic acid, benzene, or absolute alcohol, readily on heating, and dissolves with difficulty in ether.

0.1791 g. subst. (Kjeldahl); 7.9 cc. 0.1 *N* HCl.
Calc. for $C_{10}H_{10}O_3NCl$: N, 6.18%. Found: N, 6.16%.

2-Methyl-5-chloroacetylaminophenol.—20 g. 2-methyl-5-aminophenol (from monoacetyltoluylenediamine, by diazotization and boiling in the usual way) were dissolved in a mixture of 100 cc. each of acetic acid and saturated sodium acetate solution, chilled in ice-water, and treated with 19.5 cc. chloroacetyl chloride.[1] The chloroacetyl derivative separated at the end and was filtered off after the addition of an equal volume of water and allowing the mixture to stand one-half hour. The yield was 27.6 g. For analysis a portion was recrystallized successively from 50% alcohol, toluene, and glacial acetic acid, separating as rhombic plates which melt constantly at 154–5° with preliminary softening. The substance is sparingly soluble in boiling water or chloroform, dissolving readily, however, in alcohol or acetone. It is very difficultly soluble in cold acetic acid but dissolves freely on boiling. An alkaline solution couples readily with diazotized sulfanilic acid.

0.1754 g. subst. (Kjeldahl); 8.70 cc. 0.1 *N* HCl.
Calc. for $C_9H_{10}O_2NCl$: N, 7.03%. Found: N, 6.95%.

4-Methyl-5-chloroacetylaminophenol.—The 4-methyl-5-aminophenol used as starting material was prepared from the corresponding nitrotoluidine by diazotization and reduction of the resulting nitrocresol by means of tin and hydrochloric acid. It melts at 157° as stated by Bamberger and Blangey,[2] not at 144.5° as given by other workers. 12 g. of the aminocresol were dissolved in a warm mixture of 60 cc.

[1] THIS JOURNAL, 39, 1441 (1917).
[2] *Ann.*, **390**, 172, footnote (1912).

acetic acid and 60 cc. of saturated sodium acetate solution, chilled in ice-water, and treated in the usual way with 11 cc. chloroacetyl chloride. At the end the chloroacetyl derivative crystallized out and the mixture was diluted with an equal volume of water, let stand one-half hour, and filtered. After recrystallization from 50 per cent. alcohol 11.7 g. of the compound were obtained as flat, grayish, narrow plates, melting at 148–51° with preliminary softening. After two further recrystallizations from toluene the compound forms practically colorless, silky needles, which melt constantly at 151–2.5° with preliminary softening. A mixture with the 2-methyl isomer softens above 120° and melts almost entirely at 126–7° to a liquid which clears at 145°. The chloroacetyl derivative is more soluble in the usual solvents than is its 2-methyl isomer and, in aqueous suspension, gives a violet color with ferric chloride. A solution in dilute sodium carbonate couples readily with diazotized sulfanilic acid.

0.1509 g. subst. (Kjeldahl); 7.35 cc. 0.1 *N* HCl.
Calc. for $C_9H_{10}O_2NCl$: N, 7.03%. Found: N, 6.82%.

1-Chloroacetylamino-2-naphthol.—24 g. 1-amino-2-naphthol were dissolved in 180 cc. of dry acetone, chilled, and treated with 6.6 cc. chloroacetyl chloride, subsequently adding 57 cc. 2 *N* NaOH and then another 6.6 g. chloroacetyl chloride as in the case of the *o*-chloroacetylaminophenol. The mixture was finally acidified to congo-red with hydrochloric acid and diluted with water. The dark, crystalline precipitate was washed with water and recrystallized from 95% alcohol, using boneblack. The yield was 23 g. of pure material, while an additional 6.0 g. of less pure substance were recovered by concentrating the alcoholic solution and recrystallizing the product so obtained. A portion of the main fraction was recrystallized again from a small volume of 95% alcohol, separating as thin, slightly yellowish, nacreous platelets which dissolve very sparingly in boiling water. The substance is somewhat soluble in alcohol at room temperature, more easily in acetone or hot chloroform. When rapidly heated to 190°, then slowly, it melts and decomposes at 192–3°, with slight preliminary softening. In alkaline solution hydrochloric acid is readily eliminated, with formation of the anhydride of 1-amino-2-naphthoxyacetic acid, which is precipitated on acidification.

0.2100 g. subst. (Kjeldahl); 8.95 cc. 0.1 *N* HCl.
Calc. for $C_{12}H_{10}O_2NCl$: N, 5.95%. Found: N, 5.97%.

1-Chloroacetylamino-4-naphthol.—47 g. 1-amino-4-naphthol were dissolved by warming gently in a mixture of 5 parts of glacial acetic acid and 5 parts of saturated sodium acetate solution. After chilling rapidly 30.5 cc. chloroacetyl chloride were dripped in with continued cooling. The solution was then acidified to congo-red and diluted with a large volume of water, 25 g. of the chloroacetyl derivative separating. Recrystallized successively from 50% alcohol. 50% acetic acid, and 95% alcohol it forms long, faintly purplish. silky needles which soften markedly at 175–80° and melt at 199.5–201.5° to a reddish liquid. The compound dissolves in boiling water, and is quite soluble at room temperature in aclohol or acetone, somewhat less easily in chloroform. An aqueous suspension gives a violet-blue color with ferric chloride.

0.2990 g. subst. (Kjeldahl); 12.4 cc. 0.1 *N* HCl.
Calc. for $C_{12}H_{10}O_2NCl$: N, 5.95%. Found: N, 5.99%.

2,4-Dichloro-5-acetaminophenol.—53 g. *m*-acetaminophenol (from the aminophenol and acetic anhydride in dilute acetic acid solution) were dissolved by warming gently with 10 parts of glacial acetic acid. Chlorine was then passed in with constant agitation, keeping the temperature at 15–20° until the increase in weight equaled 51 g. After standing for 15 minutes the thick, crystalline slurry was diluted with an equal volume of water and the dichloro compound filtered off. The yield was 46 g. Recrystallized first from 95% alcohol, then from acetic acid it forms long, silky needles which melt at 233–6° with preliminary softening. An aqueous suspension dissolves on adding dilute sodium carbonate, while the substance also dissolves in cold alcohol or acetone, less easily in boiling chloroform or acetic acid, and quite sparingly in boiling water.

0.1630 g. subst. (Kjeldahl); 7.6 cc. 0.1 *N* HCl.
Calc. for $C_9H_9O_2NCl_2$: N, 6.37%. Found: N, 6.52%.

2,4-Dichloro-5-aminophenol.—16.5 g. of the acetamino compound were boiled for about one and one-half hours with 5 parts of 1:1 hydrochloric acid. Solution occurred gradually, followed by precipitation of the hydrochloride of the amino compound. This was

filtered off, washed with 1:1 hydrochloric acid, boneblacked in the minimum amount of hot water, and the base precipitated by adding sodium hydroxide to the filtrate. 10 g. of pure material were so obtained. Recrystallized from water, the aminophenol forms large, striated, barely cream-colored prisms which soften at 134°, melt slowly at 135–6°, and have a marked, unpleasant odor recalling that of *o*-nitrophenol. The base dissolves readily in alcohol, chloroform, or benzene; very easily in acetone or ether, and rather sparingly in cold water.

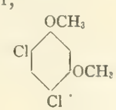
0.2305 g. subst. (Kjeldahl); 13.35 cc. 0.1 *N* HCl.
Calc. for $C_6H_5ONCl_2$: N, 7.87%. Found: N, 8.11%.

When the aminophenol is dissolved in hot 10% aqueous hydrochloric acid the hydrochloride separates on cooling as thick plates and rhombic prisms.

2,4-Dichloro-5-chloroacetylaminophenol.—This substance was prepared by the usual method,[1] using double the usual amount of 50% acetic acid and warming until the aminophenol dissolved before proceeding. The product separated immediately and was filtered off and washed with water. Recrystallized from 50% alcohol it separates as delicate, interlaced needles which melt slowly at 185.5–6.5° with preliminary softening. The compound dissolves readily in the cold in alcohol or acetone, less easily in ether or hot chloroform, and sparingly in the cold in acetic acid or toluene, dissolving readily in these solvents on boiling.

0.1748 g. subst. (Kjeldahl); 6.95 cc. 0.1 *N* HCl.
Calc. for $C_8H_6O_2NCl_3$: N, 5.50%. Found: N, 5.57%.

That the chlorine atoms in the three preceding compounds actually occupied the positions assigned to them is indicated by the series of reactions described below, by which a substance was obtained, agreeing in its melting point and other properties with 4,6-dichlororesorcin dimethyl ether,



[1] *Loc. cit.*

2,4-Dichloro-5-acetanisidide (*2,4-Dichloro-5-acetaminoanisol*).—22 g. of 2,4-dichloro-5-acetaminophenol were dissolved in 100 cc. of normal potassium hydroxide solution and shaken with 10 cc. methyl sulfate, added in small portions. The solution became opalescent with the first portion and the ether suddenly crystallized out. The process was repeated with one-half the amounts of potassium hydroxide and methyl sulfate, after which the mixture was warmed and treated with ammonia to destroy the excess of methyl sulfate. After cooling and washing with water 18 g. of the ether were obtained. Recrystallized first from alcohol, then from toluene, it forms tufts of delicate needles which melt at 157.5–9.0° with slight preliminary softening. The substance dissolves in ether or benzene, less easily in toluene or alcohol in the cold. It is very easily soluble in chloroform and very difficultly so in boiling water.

0.2287 g. subst. (Kjeldahl); 9.85 cc. 0.1 *N* HCl.
Calc. for $C_9H_9O_2NCl_2$: N, 5.99%. Found: N, 6.03%.

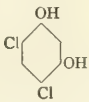
2,4-Dichloro-5-anisidine (*2,4-Dichloro-5-aminoanisol*).—16.8 g. of the acetamino compound were boiled for one-half hour under a reflux with 120 cc. 1:1 hydrochloric acid, the sparingly soluble acetyl derivative going partly into solution and then being replaced by the amino hydrochloride. After cooling in the ice-box the salt was filtered off, suspended in water, and the base precipitated with sodium carbonate, separating as an emulsion which immediately crystallized. The product was filtered off, ground up in a mortar with dilute sodium carbonate solution, filtered, and washed with water. As sodium chloride was still present the crude base was dissolved in boiling 85% alcohol, boneblacked, and the filtrate cooled and diluted with cold water until the turbidity first formed just redissolved. On seeding the base slowly crystallized as cream-colored rhombs and prisms. By further dilution of the mother-liquors 11.3 grams were obtained in all. Recrystallized from ligroin (b. 80–90°) it melts at 50.5–1.5° (corr.) with slight preliminary softening. The base has a pronounced odor, greatly resembling that of *α*-naphthylamine. It is somewhat soluble in boiling water and dissolves readily in ether, benzene, chloroform, or alcohol at room temperature. When diazotized the compound couples with R-salt to form a sparingly soluble red dye.

0.1502 g. subst. (Kjeldahl); 8.0 cc. 0.1 *N* HCl.
Calc. for $C_7H_7ONCl_2$: N, 7.30%. Found: N, 7.47%.

Constitution of 2,4-Dichloro-5-aminoanisol and Related Compounds.—3 g. of the pulverized base were added to a warm mixture of 7.8 cc. water and 5.2 cc. of concentrated sulfuric acid. The mixture was then diazotized at 10–15°, adding enough water to form a clear solution at the end. The solution was poured into a mixture of 60 cc. each of concentrated sulfuric acid and water and boiled for one hour under reflux. After standing overnight an equal volume of water and ice was added and the solution shaken with ether. The ether extract was dried over sodium sulfate and the solvent distilled off. The oily residue was then taken up in 20 cc. of normal aqueous potassium hydroxide, boneblacked, and the filtrate shaken with 1.6 cc. methyl sulfate. After the crystalline methyl ether had precipitated, an additional 10 cc. of normal potassium hydroxide solution and 8 cc. methyl sulfate were added and the mixture was again shaken and finally warmed and treated with aqueous ammonia in order to decompose the excess of methyl sulfate. The yield of crude methyl ether was 1.2 grams. Recrystallized from ligroin and then from 85 per cent. alcohol it forms faintly yellowish needles and leaflets which melt at 117–8°.

0.1025 g. subst. (Carius); 0.1409 g. AgCl.
Calc. for $C_8H_8O_2Cl_2$: Cl, 34.25%. Found: Cl, 34.02%.

There is therefore little doubt that the substance is identical with the dichlororesorcin dimethyl ether melting at 118° obtained by Auwers and Pohl[1] both by chlorinating resorcin dimethyl ether and by methylating diclororesorcin. The latter was prepared from resorcin and sulfuryl chloride, a method stated by Mettler[2] to give *para* derivatives only, and since this dichlororesorcin does not react with phthalic anhydride to give a fluorescein, *both para* positions are occupied, and the constitution is



[1] *Ann.*, 405, 279 (1914).
[2] *Ber.*, 45, 802 (1912).

From this follows the formula of the dimethyl ether, and the structure assigned above to the dichloro derivatives of *m*-aminophenol and *m*-anisidine.

2,4,6-Trichloro-5-acetaminophenol.—In the preparation of 2,4-dichloro-5-acetaminophenol it was observed that when the mother-liquors were further diluted with about an equal quantity of water and allowed to stand overnight a deposit of rhombic crystals formed. When care was taken to shake the mixture continually during the addition of the chlorine in order to avoid a local excess of the gas to as great an extent as possible, only 7.4 g. of the rhombs, which proved to be the trichloro compound, were obtained, while if the mixture was not properly agitated, more formed at the expense of the dichloro compound. Recrystallized twice from 50% alcohol containing a few drops of acetic acid the trichloro derivative forms grayish, rhombic plates containing one-half molecule of water of crystallization.. The anhydrous substance melts at 184–6.5° with preliminary softening and dissolves in alcohol, acetone, or ethyl acetate and only sparingly in boiling toluene or chloroform. The compound is appreciably soluble in boiling water and the suspension formed on cooling gives a violet color with ferric chloride. A small portion was recrystallized again from toluene and separated as rosets and sheaves of minute, flat needles, which melt slowly at 185–6.5°, with preliminary softening.

0.8485 g. air-dry subst. *in vacuo* 1st at 80°, then 100° over H_2SO_4; loss, 0.0286 g.
Calc. for $C_8H_6O_2NCl_3.1/2H_2O$: H_2O, 3.41%. Found: H_2O, 3.37%.
0.4518 g. anhydrous subst. (Kjeldahl); 17.65 cc. 0.1 *N* HCl.
Calc. for $C_8H_6O_2NCl_3$: N, 5.51%. Found: N, 5.47%.

When hydrolyzed with boiling 1:1 hydrochloric acid and neutralized with sodium acetate the compound yields 2,4,6-trichloro-5-aminophenol, which separates from ligroin as cream-colored leaflets melting at 95–6°. The compound has a bromine-like odor and crystallizes from water in colorless, hair-like needles which have the properties ascribed to the substance by Daccomo.[1]

2-Bromo-5-aminophenol.—Heller[2] prepared this substance by a devious method involving the decomposition of *p*-nitrodiazobenzene-

[1] *Ber.*, **18**, 1166 (1885).
[2] *Ibid.*, **42**, 2196 (1909).

imide with sulfuric acid, conversion of the resulting 5-nitro-2-aminophenol into the 2-bromo compound by the Sandmeyer method, reduction of this with stannous chloride, isolation of the crude base by acetylation, and final saponification of the acetyl derivative. The following direct method renders both the acetyl derivative and the base easily available. 44 g. *m*-acetaminophenol are dissolved in 260 cc. of glacial acetic acid and slowly treated with a solution of 52 g. bromine in 52 cc. acetic acid, chilling with ice. The mixture is then diluted with an equal volume of water and the resulting precipitate filtered off, washed with water, and recrystallized from 50% alcohol. 38 g. 2-bromo-5-acetaminophenol are obtained in this way, melting at 209–13° with preliminary softening. Hydrolyzed by boiling with 1:1 hydrochloric acid, neutralizing the hot liquid at the end with saturated sodium acetate solution, the acetamino compound gives an almost quantitative yield of the aminophenol. We have nothing to add to the descriptions of the two compounds as given by Heller.

2-Bromo-5-chloroacetylaminophenol.—18.5 g. 2-bromo-5-aminophenol were dissolved by warming in a mixture of 5 parts of saturated sodium acetate solution and 7 parts of glacial acetic acid. The solution was rapidly chilled and treated with 11.5 cc. chloroacetyl chloride, keeping the temperature below 10°. The chloroacetyl derivative separated immediately and was filtered off after dilution with water and washed with water and dried. The yield was 24 g. Recrystallized from alcohol it separates as rhombs which melt to a dark liquid at 191–3° with preliminary softening. It is only sparingly soluble in boiling water or chloroform and in the cold in alcohol or acetic acid, but dissolves quite readily in the latter solvents on boiling. The compound is easily soluble in acetone and dissolves in dilute aqueous ammonia or sodium carbonate. In alkaline solution it couples readily with diazotized sulfanilic acid.

0.2195 g. subst. (Kjeldahl); 8.35 cc. 0.1 N HCl.
Calc. for $C_8H_7O_2NClBr$: N, 5.30%. Found: N, 5.33%.

In synthesizing the following three phenoxyacetic derivatives the method here given for the preparation of *m*-aminophenoxyacetic acid was adopted in preference to our older method[1] because *m*-nitro-

[1] This Journal, 39, 2192 (1917).

phenol could no longer be purchased and a supply of *m*-aminophenol was at hand.

m-Acetaminophenoxyacetic Acid, *m*-$CH_3CONHC_6H_4OCH_2CO_2H$.—25 grams of *m*-acetaminophenol were suspended in 100 cc. of water and boiled for one hour in an open flask with 26.5 g. of 50 per cent. sodium hydroxide (2 mols.) and 15.5 g. of chloroacetic acid. The solution was then diluted, treated with one-half the original amount of alkali and chloroacetic acid, and boiled until the reaction became acid. On acidifying strongly with hydrochloric acid the acetaminophenoxyacetic acid separated as an oil which quickly solidified when rubbed. The yield was good. Recrystallized from water it forms delicate needles containing one molecule of water of crystallization. When plunged into a bath at 165° the air-dry substance melts with effervescence; the anhydrous compound melts slowly at 170.5–2.5° with preliminary softening, the figures being unaffected by a subsequent recrystallization from acetic acid. The air-dry acid is sparingly soluble in cold water, readily on boiling; the anhydrous substance dissolves readily in dry methyl alcohol, less easily in absolute alcohol or dry acetone, and very sparingly in boiling toluene. It dissolves readily in boiling acetic acid, separating on cooling as spherules of microcrystals.

0.3966 g. air-dry subst. *in vacuo* at 100° over H_2SO_4; loss, 0.0322 g.
Calc. for $C_{10}H_{11}O_4N.H_2O$: H_2O, 7.93%. Found: H_2O, 8.12%.
Anhydrous: 0.1508 g. subst. (Kjeldahl); 7.2 cc. 0.1 *N* HCl.
Calc. for $C_{10}H_{11}O_4N$: N, 6.70%. Found: N, 6.69%.

When boiled for one and one-half hours with 1:1 hydrochloric acid and cooled the acetamino compound readily yields *m*-aminophenoxyacetic acid hydrochloride, which separates from the solution. The free acid obtained from this after dissolving in hot water, boneblacking, and neutralizing with sodium acetate agreed in melting point and all its other properties with the *m*-aminophenoxyacetic acid prepared by the reduction of the nitro compound.[1]

m-Chloroacetylaminophenoxyacetic Acid, *m*-$ClCH_2CONHC_6H_4OCH_2$-CO_2H.—25 grams of *m*-aminophenoxyacetic acid were dissolved in a warm mixture of 250 cc. of glacial acetic acid and 250 cc. of satu-

[1] *Loc. cit*

rated sodium acetate solution. After rapid chilling the solution was treated with 18 cc. of chloroacetyl chloride, stirring vigorously, and keeping the temperature about 0°. The resulting clear solution was concentrated *in vacuo* to a syrup, taken up in water, and treated with hydrochloric acid (1:1) until acid to congo-red. The chloroacetyl compound separated on scratching, and after standing in the ice-box overnight, was filtered off and washed with water. The crude product was recrystallized from water with the aid of boneblack, separating as an oily suspension which readily crystallized on rubbing. After thorough cooling the acid was filtered off, washed, and dried. The yield was 31 grams. A portion was recrystallized from acetic acid, adding an equal volume of water to the solution, cooling, seeding, and letting stand in the ice-box. The acid separated slowly as spherular aggregates of microscopic crystals which are soluble in the cold in alcohol or acetone, less easily in acetic acid, and dissolve very sparingly in cold water but readily on boiling. The substance melts with preliminary softening at 159–60° to a turbid liquid which clears at 162°.

0.2040 g. subst. (Kjeldahl); 8.2 cc. 0.1 *N* HCl.
Calc. for $C_{10}H_{10}O_4NCl$: N, 5.75%. Found: N, 5.63%.

In the following synthesis of 4-aminopyrocatechol, the hydrochloride of which has been prepared by Benedikt[1] by a different method, 4-amino-guaiacol was required in considerable amount. Of the methods available for the preparation of this substance that of Rupe,[2] namely, coupling diazotized sulfanilic acid with guaiacol and reducing the sodium salt of the dye with tin and hydrochloric acid, seemed best suited for preparative purposes, although Rupe states that the method did not result in a pure product. It was found that by isolating the free sulfonic acid and reducing this with hydrogen sulfide in ammoniacal solution 4-aminoguaiacol could easily be obtained pure and in quantity.

p-Sulfophenylazoguaiacol.—104 g. sulfanilic acid (dry weight) were dissolved in 600 cc. of normal aqueous sodium hydroxide and diluted with ice and water to about 2.5 liters. After adding 45 g.

[1] *Ber.*, 11, 363 (1878).
[2] *Ibid.*, 30, 2447 (1897).

sodium nitrite the solution was turbined and treated slowly with 360 cc. of 1:1 hydrochloric acid. When diazotization was complete the resulting slurry was slowly poured into a solution of 77 g. guaiacol in 2400 cc. of normal sodium hydroxide containing ice. After stirring for three-quarters of an hour about one-quarter volume of concentrated hydrochloric acid was added, whereupon the dye separated on rubbing and letting stand. The product was filtered off and washed with 1:1 hydrochloric acid and acetone. The yield of crude dye, sufficiently pure for the subsequent reduction, was 140 g. Recrystallized from water the sulfonic acid separates as glistening, metallic green needles and long, thin plates, appearing orange under the microscope. When heated, decomposition begins above 220° and becomes rapid at about 245°. The dye dissolves in water with an orange-yellow color, deepening to brown on adding ferric chloride. It is insoluble in chloroform and almost so in acetic acid or acetone, but turns deep red on boiling with acetic acid, possibly due to elimination of water of crystallization, although there was no loss on heating the air-dry substance at 100° *in vacuo* over sulfuric acid.

0.1423 g. subst.; 10.4 cc. N (22.0°, 768 mm.)
Calc. for $C_{13}H_{12}O_6N_2S$: N, 9.09%; + $1H_2O$: N, 8.56%. Found: N, 8.54%.

4-Aminoguaiacol.—140 g. of the crude sulfo dye were dissolved in 1400 cc. of 10% aqueous ammonia and saturated with a rapid stream of hydrogen sulfide. The solution became hot, the color suddenly changed to yellow, and crystals of the aminoguaiacol soon separated. After letting stand for an hour and chilling, the substance was filtered off and washed with water. The yield was 48 g., melting at 177–8° with preliminary darkening, and corresponding to the recorded properties of this base.

4-Aminopyrocatechol Hydrobromide.—85 g. 4-aminoguaiacol were boiled under a reflux condenser with 5 parts of hydrobrominic acid (d. 1.49) for 6 hours. The crystals which separated on cooling were boiled again for 3 hours with fresh hydrobromic acid (b. 123°), while the mother-liquors were also boiled an additional 3 hours. On chilling a total of 90 g. of dark crystals was obtained. A portion of the salt was purified by dissolving in water to which a few drops of hydrobromic acid had been added, adding a little stannous chloride

to the deep green solution, and passing in hydrogen sulfide. The precipitate was filtered off and the solution concentrated *in vacuo* to small bulk and let stand in the ice-box. The almost colorless crystals which separated were recrystallized from 85% alcohol, adding a little concentrated hydrobromic acid before cooling. On seeding, the hydrobromide separated slowly as flat needles or long, narrow plates. The salt was washed with 95% alcohol containing a little hydrobromic acid and dried *in vacuo* over sulfuric acid and crushed potassium hydroxide. The compound darkens somewhat above 150° and melts with decomposition at about 255–60°, with preliminary softening and darkening. It is readily soluble in water, the solution giving a cherry-red color with ferric chloride, changing to red-brown and finally forming a violet-black precipitate. It is easily soluble at room temperature in alcohol of various strengths, but is less soluble in alcohol at 0° in the presence of hydrobromic acid.

0.1675 g. subst. (Kjeldahl); 8.25 cc. 0.1 N HCl.
0.1186 g. subst. (Carius); 0.1074 g. AgBr.
Calc. for $C_6H_7O_2N.HBr$: N, 6.81; Br, 38.79%. Found: N, 6.90; Br, 38.55%.

4-Aminopyrocatechol (3,4-dihydroxyaniline). A portion of the recrystallized hydrobromide was placed in a bottle and the air displaced by a current of carbon dioxide. A little water was added through a dropping funnel, followed, when the salt had dissolved, by a saturated sodium bicarbonate solution. On rubbing for a moment the base separated from the pink solution as colorless prisms, often with key-like ends. The aminophenol was filtered off, directing a stream of carbon dioxide on to the funnel, and rapidly washed with several small portions of ice-water, but these precautions were not sufficient entirely to prevent darkening of the sensitive base. After rapidly transferring to a dish, keeping a stream of carbon dioxide playing on the substance, it was dried in a high vacuum. As so obtained the aminophenol melts at 122–3° with preliminary softening and darkening, evolving gas, and forming a dark purple mass. It is quite soluble in water at room temperature, except for a slight, dark, amorphous residue of oxidation products which increases as the solution is allowed to stand. It dissolves in 95% alcohol, less easily in acetone, and very sparingly in boiling benzene or toluene. When recrystallized again by dissolving in a little boiling absolute alcohol,

adding an equal volume of benzene, and rubbing, the substance separates quickly as gray plates and clusters of short prisms which melt at 124–5° with the same phenomenon as described above.

0.1415 g. subst. (Kjeldahl); 11.45 cc. 0.1 *N* HCl.
Calc. for $C_6H_7O_2N$: N, 11.21%. Found: N, 11.33%.

4-Chloroacetylaminopyrocatechol.—50 g. of the crude aminopyrocatechol hydrobromide were dissolved in 300 cc. water and treated with 92 g. of crystalline sodium acetate. The mixture, from which a portion of the free aminopyrocatechol had separated, was chilled in ice-water, vigorously stirred, and treated, drop by drop, with 30 cc. chloroacetyl chloride. Deposition of the chloroacetyl derivative began before the base had entirely dissolved. At the end the mixture was made strongly acid to congo-red with hydrochloric acid and the precipitate filtered off, washed with water, and dried. The yield of crude product was 38 g. Recrystallized successively from water, acetic acid, and ethyl acetate, it forms flat, delicate needles which melt at 156–7.5° with preliminary softening. The compound is sparingly soluble in cold water, readily on boiling, the aqueous solution giving an olive-brown color with ferric chloride. It dissolves easily in alcohol and sparingly in the cold in acetic acid or ethyl acetate, easily on boiling.

0.1647 g. subst. (Kjeldahl); 8.40 cc. 0.1 *N* HCl.
Calc. for $C_8H_8O_3NCl$: N, 6.95%. Found: N, 7.14%.

p-Chloroacetylaminoacetophenone.—A well chilled solution of 16 g. *p*-aminoacetophenone in 160 cc. of dry acetone was treated slowly with 10 cc. of chloroacetyl chloride, following this slowly with 50 cc. of 10% sodium hydroxide solution. After acidification with hydrochloric acid the mixture was diluted with water, causing the separation of the chloroacetyl compound. After recrystallization from 50% alcohol it was obtained as a woolly mass of needles. The yield was 16 g. Recrystallized again from toluene the compound forms arborescent aggregates of plates which melt at 152–3° (corr.) with slight preliminary softening. It is sparingly soluble in ether or hot benzene and readily so in alcohol or hot toluene.

0.2663 g. subst. (Kjeldahl); 12.45 cc. 0.1 *N* HCl.
Calc. for $C_{10}H_{10}O_2NCl$: N, 6.62%. Found: N, 6.55%.

p-Chloroacetylaminophenylacetic Acid, *p*-$ClCH_2CONHC_6H_4CH_2$-CO_2H.—8.5 g. *p*-aminophenylacetic acid were suspended in 100 cc. of 20% sodium acetate solution and treated with dilute sodium hydroxide until solution was complete. After adding ice, 8 cc. chloroacetyl chloride were dropped in, with shaking. The solution was acidified with hydrochloric acid and the chloroacetylamino acid filtered off, washed, and dried. The yield was 10 g. Recrystallized successively from water, toluene, and acetic acid it separates as faintly yellow, glistening leaflets which melt at 158–60° to a yellow liquid, with preliminary softening. A solution of the acid in boiling water deposits the substance on cooling as snowy, arborescent masses of delicate needles. It dissolves in alcohol, more easily in acetone, and also dissolves in boiling acetic acid and only sparingly in boiling toluene.

0.2109 g. subst. (Kjeldahl); 9.45 cc. 0.1 *N* HCl.
Calc. for $C_{10}H_{10}O_3NCl$: N, 6.16%. Found: N, 6.28%.

Ethyl Chloroacetylanthranilate, *o*-$ClCH_2CONHC_6H_4CO_2C_2H_5$.—20 g. ethyl anthranilate hydrochloride, 100 cc. toluene, and 100 cc. of normal sodium hydroxide solution were shaken together until all the ester had gone into solution in the toluene. 100 cc. of 10% sodium hydroxide solution were then added, followed by 14 cc. chloroacetyl chloride, drop by drop, with shaking and cooling, adding more alkali to maintain the alkalinity toward the end. The toluene layer was dried over calcium chloride, evaporated on the water bath, and the residue recrystallized from 95% alcohol. The yield was 23 g. Recrystallized from 85% alcohol the chloroacetyl ester forms glistening needles which melt at 79.5–80° (corr.) and dissolve readily in benzene, toluene, or chloroform, somewhat less so in 95% alcohol.

0.3452 g. subst. (Kjeldahl); 13.6 cc. 0.1 *N* HCl.
Calc. for $C_{11}H_{12}O_3NCl$: N, 5.80%. Found: N, 5.52%.

Ethyl Iodoacetylanthranilate.—10 g. of the chloroacetyl ester were suspended in 25 cc. acetone, treated with 50 cc. of a normal solution of sodium iodide in dry acetone,[1] and the mixture was warmed until solution was complete. Deposition of sodium chloride soon commenced, and after standing overnight water was added, precipitating the

[1] Finkelstein, *Ber.*, 43, 1528 (1910).

iodoacetyl compound. The yield was 13 g. after recrystallizing from 95% alcohol with the aid of a freezing mixture. Recrystallized again from 85% alcohol, in which it is less soluble, it formed glistening, transparent prisms which soften at 78° and melt at 78.5–9.0° (corr.). The compound is easily soluble in the cold in chloroform or toluene.

0.4483 g. subst. (Kjeldahl); 13.05 cc. 0.1 *N* HCl.
Calc. for $C_{11}H_{12}O_3NI$: N, 4.21%. Found: N, 4.08%.

Chloroacetyl-N-methylanthranilic Acid, o-$HOOCC_6H_4N(CH_3)COCH_2$-Cl.—4.5 g. methylanthranilic acid were dissolved in 40 cc. of normal aqueous hydroxide, 10 cc. of saturated sodium acetate solution added, and then 4 cc. chloroacetyl chloride, with chilling and shaking. The reaction mixture was next acidified to congo-red with hydrochloric acid. The oil which separated crystallized on rubbing, and the yield was practically quantitative. Recrystallized from toluene the substance forms colorless spears which melt at 167–8° (corr.) with slight preliminary sintering. It is readily soluble in alcohol, acetone, or hot water.

0.3107 g. subst. (Kjeldahl); 13.55 cc. 0.1 *N* HCl.
Calc. for $C_{10}H_{10}O_3NCl$: N, 6.16%. Found: N, 6.11%.

Ethyl Chloroacetylmethylanthranilate, o-$ClCH_2CON(CH_3)C_6H_4CO_2$-$C_2H_5$.—This substance was prepared from ethyl methylanthranilate (from the acid with alcohol and sulfuric acid) by the method used for the preparation of ethyl chloroacetylanthranilate. The residue from the evaporation of the toluene slowly crystallized and when dissolved in boiling petroleum ether it separated on cooling as stout, colorless prisms which melt at 50–1° (corr.) and are easily soluble in the usual organic solvents except petroleum ether. 16 g. ethyl methylanthranilate yielded 15 g. of the purified acyl derivative.

0.2940 g. subst. (Kjeldahl); 11.25 cc. 0.1 *N* HCl.
Calc. for $C_{12}H_{14}O_3NCl$: N, 5.48%. Found: N, 5.36%.

Sodium Chloroacetylsulfanilate, p-$NaO_3SC_6H_4NHCOCH_2Cl$.—49 g. sodium sulfanilate were dissolved in 10 parts of water, 25 g. sodium carbonate were added, and the solution chilled in a freezing mixture and stirred. When the temperature had dropped to $-2°$ the dropwise addition of 30 cc. chloroacetyl chloride was commenced. Toward

the end the mixture set to an almost solid mass, after which the freezing mixture was removed and the turbining continued until the mixture again became fluid. The rest of the chloroacetyl chloride was then added drop by drop without further cooling. 150 g. salt were then added, with continued stirring, causing the sudden precipitation of the chloroacetylsulfanilate. The product was filtered off, washed with 85% alcohol, and recrystallized from this solvent, giving 23 g. of crude substance still containing appreciable amounts of sodium chloride. A pure product was obtained for analysis, however, by washing the product with small portions of ice-water, then with 50% alcohol, and finally dissolving in a little warm water, filtering, and adding several volumes of alcohol. As so obtained the salt separates as radiating masses of hair-like needles which contain no water of crystallization when air-dried and which dissolve readily in water. The aqueous solution gives only a faint turbidity with silver nitrate in the cold, but if the salt is dried at 100° partial decomposition sets in and a copious precipitate of silver chloride is obtained. The free acid was not isolated in a state of purity.

0.1543 g. subst. (Kjeldahl); 5.65 cc. 0.1 N HCl.
Calc. for $C_8H_7O_4NClSNa$: N, 5.16%. Found: N, 5.13%.

4-Chloroacetylamino-6-hydroxybenzenesulfonic Acid and Its Sodium Salt.—4-Amino-6-hydroxybenzenesulfonic acid (*m*-aminophenolsulfonic acid) was easily obtained in good yield by gradually adding *m*-aminophenol to 4 parts of concentrated sulfuric acid, with occasional cooling, and heating the solution on the water bath for one hour. The precipitate obtained by pouring on to ice was collected, washed well with water, suspended in hot water, and dissolved by adding sodium acetate. The hot solution was boneblacked and the free acid precipitated from the filtrate by means of hydrochloric acid. 36 g. of the 4-amino-6-hydroxybenzenesulfonic acid were dissolved in a mixture of 190 cc. of normal sodium hydroxide solution, 190 cc. water, and 19 g. sodium carbonate. The solution was stirred, chilled to 5°, and treated with 22.5 cc. chloroacetyl chloride. About 100 g. sodium chloride were then added, causing the precipitation of the sodium salt of the acyl sulfonic acid. After stirring for 10 minutes the salt was filtered off, washed with a little ice-water, then

with 85% alcohol, and dried *in vacuo* over sulfuric acid. The yield was 47 g. A portion was dissolved in the minimum amount of hot water, and the solution filtered and treated with several volumes of alcohol. On seeding, the salt separated rapidly as a voluminous mass of plumes of minute, hair-like needles which contain one-half molecule of water of crystallization. The salt is quite readily soluble in water, the solution giving a purple color with ferric chloride. An alkaline solution couples readily with diazotized sulfanilic acid.

0.4926 g. air-dry subst. *in vacuo* at 80° over H_2SO_4; loss, 0.0134 g.
Calc. for $C_8H_7O_6NClSNa.1/2H_2O$: H_2O, 3.04%. Found: H_2O, 2.74%.
0.1441 g. anhydrous subst. (Kjeldahl); 5.1 cc. 0.1 *N* HCl.
Calc. for $C_8H_7O_6NClSNa$: N, 4.87%. Found: N, 4.96%.

The *free acid* was obtained by dissolving a portion of the salt in hot 10% hydrochloric acid, filtering, and cooling, rapid manipulation being necessary in order to avoid saponification of the acyl group. On seeding with crystals obtained from a similarly treated preliminary experiment (1:1 acid was used) which had been allowed to stand in the ice-box, the acid separated slowly at 0° as minute platelets and flat needles which apparently contain no water of crystallization. When heated, the acid darkens slightly, but does not melt below 275°. It is sparingly soluble in cold water, but dissolves quite easily on boiling. Boiling absolute alcohol or acetic acid dissolves only traces.

0.1548 g. subst. (Kjeldahl); 5.95 cc. 0.1 *N* HCl.
0.2370 g. subst. (hydrolysis with NaOH); 0.1258 g. AgCl.
Calc. for $C_8H_8O_6NClS$: N, 5.28; Cl, 13.35%. Found: N, 5.39; Cl, 13.13%.

With the exception of the hexamethylenetetramine compound, which was used in another connection, the aliphatic substances discussed below also figured as intermediates in the work on arsenic compounds mentioned in the introduction.

Chloroacetmethylamide, $ClCH_2CONHCH_3$.—Since the original description of this substance[1] it has been found that in working up larger amounts of material it is advantageous to use a mechanical stirrer and to add only one-half of the required amount of alkali at the beginning, deferring the addition of the remainder until almost one-half of the chloroacetyl chloride has been added. At the end,

[1] *J. Biol. Chem.*, 21, 147 (1915).

after neutralization, the solution is partially saturated with salt to diminish the solubility of the chloroacetmethylamide and shaken out 8 times with chloroform. After drying this over sodium sulfate and concentrating to small bulk the amide is recovered by distillation *in vacuo*, the slight loss due to the volatility of the compound being more than offset by the time saved. In this way, starting with 78 g. methylamine hydrochloride 70 g. of the chloroacetmethylamide were obtained, boiling at 112–3° under 22 mm. pressure and solidifying at once in the receiver.

Chloroacet-n-propylamide, $ClCH_2CONHC_3H_7$.—10 g. *n*-propylamine were added to 125 cc. of 10 per cent. sodium hydroxide solution. To this mixture, which was turbined and chilled in a freezing mixture, were added, drop by drop, 13.3 cc. chloroacetyl chloride dissolved in 30 cc. toluene. The mixture was finally acidified to congo-red with hydrochloric acid and extracted with chloroform. The chloroform extract was dried over calcium chloride and concentrated, the oily residue being fractionated *in vacuo*. The main fraction (12 grams) boiled at 107–8° at 13 mm. On redistillation it boiled at 105–6° (corr.) at 10.5 mm. It is a rather viscous oil which dissolves in water but does not mix with it in all proportions. It is miscible with alcohol, acetone, chloroform, and ether.

0.1828 g. subst. (Kjeldahl); 13.35 cc. 0.1 *N* HCl.
Calc. for $C_5H_{10}ONCl$: N, 10.33%. Found: N, 10.23%.

Hexamethylenetetramonium Salt of Chloroacet-n-propylamide.—2.5 g. each of chloroacetpropylamide and hexamethylenetetramine were boiled with dry chloroform for one hour. The resulting solution was filtered from a trace of precipitate and treated with 2–3 volumes of dry acetone, whereupon the salt separated as thick, hexagonal platelets and relatively few delicate needles. The yield was 3.2 g. The salt dissolves readily in water, alcohol, methyl alcohol, or chloroform, and is practically insoluble in dry acetone. When rapidly heated to 145°, then slowly, it melts to a dark liquid at 147–9°.

0.1566 g. subst.; 10.98 cc. $AgNO_3$ soln. (1 cc. = 0.001794 g. Cl).
Calc. for $C_{11}H_{22}ON_5Cl$: Cl, 12.86%. Found: Cl, 12.58%.

Chloroacetylethylurea, $ClCH_2CONHCONHC_2H_5$.—10 g. ethylurea and 14 g. chloroacetyl chloride (1.1 mols.) were boiled in 100 g. of dry

benzene for one hour. Water was added to the mixture, which was then filtered. The solid was recrystallized from 50% alcohol, 14 g. of the ureide being obtained. For analysis a portion was recrystallized from benzene, forming long needles which melt at 141.5–2.5° (corr.). The compound dissolves in acetone, chloroform, or alcohol, and only sparingly in cold benzene.

0.1820 g. subst. (Kjeldahl); 22.2 cc. 0.1 *N* HCl.
Calc. for $C_5H_9O_2N_2Cl$: N, 17.02%. Found: N, 17.09%.

Chloroacetpiperidide, $ClCH_2CONC_5H_{10}$.—In the original method given for the preparation of this substance[1] a poor yield was obtained owing to its not having been foreseen that the piperidide would be soluble in water. A satisfactory yield may be achieved as follows: 25 g. piperidine are dissolved in 150 cc. of dry, alcohol-free ether and cooled to about − 5°. The solution is shaken vigorously and kept below 0° while a solution of 12 cc. chloroacetyl chloride in dry, alcohol-free ether is dropped in. After letting stand for a short time the precipitate or piperidine hydrochloride is filtered off, washed with dry ether, and the filtrate and washings are concentrated to small bulk. 16.3 g. chloroacetpiperidide are recovered from the residue, the boiling point being 149–53° under a pressure of 17 mm. Most of the substance boils at 151°.

The following table of compounds is appended in the order described:

[1] *J. Biol. Chem.*, **21**, 150 (1915).

Name.	Formula.	Melting point. °C.
o-Chloroacetylaminophenol	$C_8H_8O_2NCl$	138–40
o-Chloroacetylaminophenyl acetate	$C_{10}H_{10}O_3NCl$	113.5–4.5
2-Methyl-5-chloroacetylaminophenol	$C_9H_{10}O_2NCl$	154–5
4-Methyl-5-chloroacetylaminophenol	$C_9H_{10}O_2NCl$	151–2.5
1-Chloroacetylamino-2-naphthol	$C_{12}H_{10}O_2NCl$	192–3
1-Chloroacetylamino-4-naphthol	$C_{12}H_{10}O_2NCl$	199.5–201.5
2,4-Dichloro-5-acetaminophenol	$C_8H_7O_2NCl_2$	233–6
2,4-Dichloro-5-aminophenol	$C_6H_5ONCl_2$	135–6
2,4-Dichloro-5-chloroacetylaminophenol	$C_8H_6O_2NCl_3$	185.5–6.5
2,4-Dichloro-5-acetaminoanisol	$C_9H_9O_2NCl_2$	157.5–9.0
2,4-Dichloro-5-anisidine	$C_7H_7ONCl_2$	50.5–1.5
2,4-Dichlororesorcin dimethyl ether	$C_8H_8O_2Cl_2$	117–8
2,4,6-Trichloro-5-acetaminophenol	$C_8H_6O_2NCl_3$	185.5–6.5
2,4,6-Trichloro-5-aminophenol	$C_6H_4ONCl_3$	95–6
2-Bromo-5-aminophenol	C_6H_6ONBr	
2-Bromo-5-chloroacetylaminophenol	$C_8H_7O_2NClBr$	191–3
m-Acetaminophenoxyacetic acid	$C_{10}H_{11}O_4N$	170.5–2.5
m-Aminophenoxyacetic acid	$C_8H_9O_3N$	
m-Chloroacetylaminophenoxyacetic acid	$C_{10}H_{1}\ O_4NCl$	159–62
p-Sulfophenylazoguaiacol	$C_{13}H_{12}O_5N_2S$	
4-Aminoguaiacol	$C_7H_9O_2N$	177–8
4-Aminopyrocatechol hydrobromide	$C_6H_7O_2N.HBr$	255–60
4-Aminopyrocatechol	$C_6H_7O_2N$	124–5
4-Chloroacetylaminopyrocatechol	$C_8H_8O_3NCl$	156–7.5
p-Chloroacetylaminoacetophenone	$C_{10}H_{10}O_2NCl$	152–3
p-Chloroacetylaminophenylacetic acid	$C_{10}H_{10}O_3NCl$	158–60
Ethyl chloroacetylanthranilate	$C_{11}H_{12}O_3NCl$	79.5–80
Ethyl iodoacetylanthranilate	$C_{11}H_{12}O_3NI$	78.5–9.0
Chloroacetyl-*N*-methylanthranilic acid	$C_{10}H_{10}O_3NCl$	167–8
Ethyl chloroacetylmethylanthranilate	$C_{12}H_{14}O_3NCl$	50–1
Sodium chloroacetylsulfanilate	$C_8H_7O_4NClSNa$	
4-Chloroacetylamino-6-hydroxybenzenesulfonic acid	$C_8H_8O_5NClS$	
Sodium 4-chloroacetylamino-6-hydroxybenzenesulfonate	$C_8H_7O_5NClSNa$	
Chloroacetmethylamide	C_3H_6ONCl	b_{22} 112–3
Chloroacet-*n*-propylamide	$C_5H_{10}ONCl$	$b_{10.5}$ 105–6
Hexamethylenetetramonium salt	$C_{11}H_{22}ON_5Cl$	147–9
Chloroacetylethylurea	$C_5H_9O_2N_2Cl$	141.5–2.5
Chloroacetpiperidide	$C_7H_{12}ONCl$	b_{17} 151

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CICATRIZATION OF WOUNDS.

X. A GENERAL EQUATION FOR THE LAW OF CICATRIZATION OF SURFACE WOUNDS.

BY P. LECOMTE DU NOÜY, D.Sc.

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York, and Hospital 21, Compiègne, France.*)

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It has been shown[1] by the extrapolation formula

$$S_n = S_{n-1}\left[1 - i\,(t + \sqrt{t + nt})\,\right]$$

that the normal progress of cicatrization of surface wounds follows a definite curve. The fact that many biological and chemical phenomena are expressed by exponential formulas suggested the comparison, if possible, of the curve for the cicatrization of wounds with other curves expressing biological phenomena. It is well known that the exponential function plays an important part in natural phenomena. It expresses the general law called by Lord Kelvin "the compound interest law," and by Mellor, the "ubiquitous law."

I had already studied a formula of the form

$$y = \frac{K}{x - a} \quad \text{(hyperbola)}$$

which was suggested to me by Professor Houssay, by means of which he expresses the phenomenon of regression of certain organs in animals, under special conditions; but this proved to be unsuccessful.

On the basis that during the short time dt the cicatrized area ds remains proportional to the total area, we can write

$$-\,ds = KSdt \qquad (1)$$

[1] du Noüy, P. L., *J. Exp. Med.*, 1916, xxiv, 451, 461; 1917, xxv, 721.

by integration in respect to time,

(2)
$$T = -\int_{S_o}^{S} \frac{ds}{KS}$$

or

$$T = -\frac{1}{K}\int_{S_o}^{S} \frac{ds}{S}$$

hence

$$T = \frac{1}{K} \text{ Log}_e \frac{S_o}{S}$$

which is similar to the equation of Slater,[2]

$$T = \frac{1}{K} \text{ Log}_e \frac{N+n}{N}$$

and finally,

(3)
$$KT = \text{Log}_e \frac{S_o}{S}$$

that is,

$$S = S_o e^{-KT}$$

We can then compute the values of the coefficient K for the different values of T. K increases regularly. Therefore, the curve obtained from the equation

$$S = S_o e^{-KT}$$

does not correspond to the facts, and gives for every value of T a certain value of S which deviates more and more from that calculated according to formula (1) (extrapolation form). We were then obliged to introduce a new coefficient, stating the problem in the following way: Is it better to attempt to find this new coefficient by giving to T its real value and by studying the variations of K, or is it more advisable to study the variations of the exponent if K remains

[2] Slater, A., *Biochem. J.*, 1912–13, vii, 197.

constant; that is, the variations of a certain coefficient α as in the exponent

$$-K(T+\alpha) \tag{4}$$

The study of a large number of cases showed that by trying to find the correction of the coefficient K, I encountered a practical difficulty from the fact that since this coefficient is small in respect to T, the smallest numerical variations such as those arising from calculation errors with 2 or 3 decimal numbers were of sufficient importance to destroy the concordance of the curves. On the contrary, in the second case, fairly important variations in a certain coefficient K_2, the connection of which with α can be expressed as

$$\alpha = \frac{T^2}{K_2}, \tag{5}$$

interfered very little with the accuracy of the calculation.

Text-fig. 1 shows the variations of the coefficient K in function of time. The angular coefficient of the lines seems to vary proportionally with the index of cicatrization, as

$$\text{index } i = \frac{S_o - S}{S_o\,(t + \sqrt{t})}$$

It is by no means certain that these lines are straight lines mathematically (see the straight dotted line in Text-fig. 1), but the observations are limited by time and it is difficult to determine this point. In this chart the value of K is given by equation (3) from which the following formula is obtained:

$$K = \frac{\operatorname{Log} S_o - \operatorname{Log} S}{T} \tag{6}$$

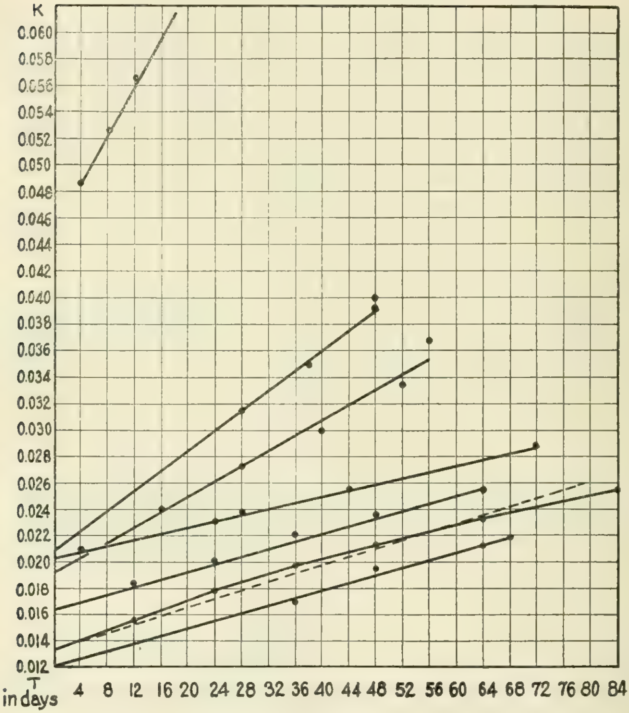
Text-fig. 2, on the contrary, shows the variations of the coefficient α previously determined, the value of which is

$$\alpha = \frac{\operatorname{Log} S_o - \operatorname{Log} S}{K} - T \tag{7}$$

By plotting in ordinates the values of α which represent the difference between the curve resulting from equation (3) and that resulting

from equation (1), we obtain a curve which expresses the law of these differences. It is a branch of parabola and the equation is

$$y^2 = 2\ px$$



TEXT-FIG. 1. Variations of the coefficient K, in function of time.

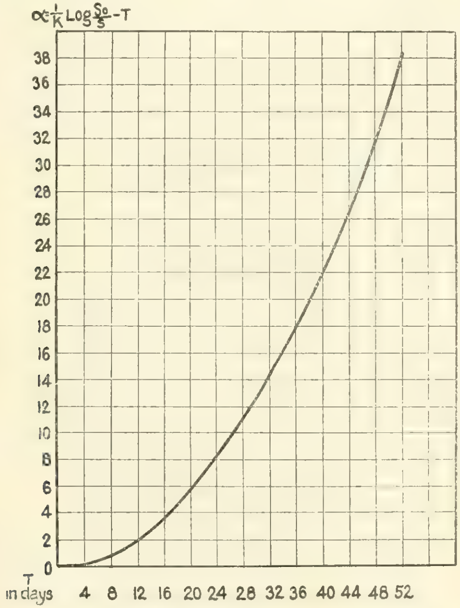
That is, by replacing the letters by those we have adopted, *viz.*, $y = T$, and $x = \alpha$,

$$\alpha = \frac{T^2}{2p} \tag{8}$$

The significance of the coefficient K_2 in equation (5) appears now clearly, and the equation may be written

$$S_T = S_o e^{-K\left(T + \frac{T^2}{2p}\right)} \tag{9}$$

which is the general equation of the law.



TEXT-FIG. 2. Variations of the coefficient α in function of time in the formula

$$S_T = S_o e^{-K(T+\alpha)}$$

Before we begin a thorough study of the coefficients it may be interesting to compare, for example, two series of figures representing the ordinates, *viz.* the areas of wounds in square centimeters, of two

cicatrization curves obtained, the first (figures of the upper row) by means of the last exponential equation (9), the second (figures of the lower row) by means of the former extrapolation formula (1). It is obvious that the concordance is almost perfect and that the differences are beyond the errors of experimentation (Table I).

These two examples suffice to show that the proposed equation fulfills the required conditions; in all the cases the coincidence is equally satisfactory. Slight differences, however, sometimes may be observed at the beginning of the curve (for $T = 4, 8, 12$ days), but since the exponential equation has been mathematically studied in a different manner from the first formula, and since, on the other hand, these differences may be affected by errors of measure of the area of wounds, it cannot be concluded that the equation previously proposed is more accurate than the new one.

Study of the Coefficients K and 2p.

As the coefficient K can be determined within 4 days, that is from two points on the curve, 4 days apart, and as the contraction, especially for the large wounds, plays the principal part at the beginning of cicatrization, this coefficient characterizes the contraction, and during the first days the relative rate of repair, with reference to the total area of the wound. But it has been stated[1] that this rate is itself a function of the age of the man, within certain limits. Hence the coefficient K must logically be proportional to the index of cicatrization i which plays the same part in formula (1). The calculation of a number of curves shows that this is so.

The velocity of repair is originally determined by the area of the wound. We have assumed that at the beginning of the phenomenon it remained proportional to the area for a very short time. We proceeded from this assumption to state the differential equation

$$-ds = KSdt$$

If the velocity remained proportional to the area, this would explain the increasing delay due to the reduction of the area of the wound. On account of this delay, the phenomenon is expressed by a logarithmic curve and not by a straight line, for, at a certain moment T, the area which is not yet cicatrized is $TM = S$ (Text-fig. 3).

TABLE I.

Area calculated with.	Time in days.																			
	0	4	8	12	16	20	24	28	32	36	40	44	48	52	56	60	64	68	72	76
Patient 263.																				
	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.	sq. cm.
Equation (9)	61.8	50.8	41.4	33.4	26.8	21.2	16.7	13.1	10.2	7.7	6.0	4.5	3.4	2.5	1.8	1.4	1.0	0.7	0.5	0.4
" (1)	61.8	51.0	41.6	33.6	26.9	21.3	16.7	13.1	10.2	7.7	6.0	4.5	3.4	2.5	1.8	1.4	1.0	0.7	0.5	0.4
Patient 706.																				
Equation (9)	27.4	21.8	17.1	13.4	9.9	7.2	5.1	3.6	2.4	1.6	1.1	0.69	0.43							
" (1)	27.4	22.0	17.1	13.0	9.6	7.2	5.1	3.6	2.4	1.6	1.1	0.7	0.46							

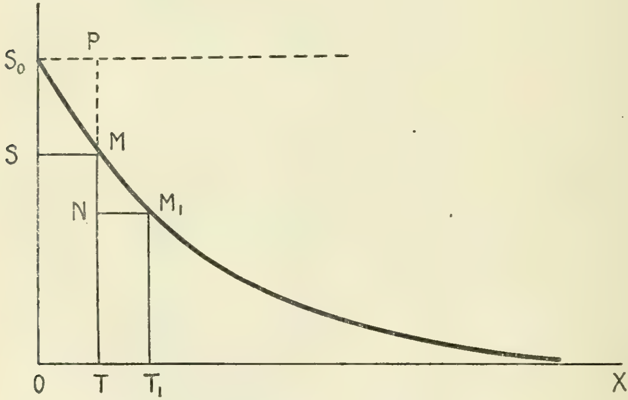
The surface which is already cicatrized is represented by

$$MP = S_0 - S$$

The law of the curve, if logarithmic, is that the decrease MN of the ordinate S, when passing from the time T to the time T_1, is proportional to the length S of the ordinate; that is, the area cicatrized during the time $T_1 - T$ is proportional to the area which is not yet cicatrized. This is what we have written in mathematical symbols, for infinitesimal values

$$-ds = KSdt$$

for ds corresponds to MN and dt to $T_1 - T$.



TEXT-FIG. 3. Logarithmic curve.

But this hypothesis, true at the beginning of the phenomenon, under certain conditions, grows rapidly erroneous, since we have stated that the curve resulting from this equation deviates more and more from the experimental facts. Hence the diminution of the area is not the only factor which governs the real curve. A careful study of the latter and a comparison with the plain logarithmic curve shows that to the *decreasing* acceleration a uniformly *increasing* acceleration is opposed, which at every moment counteracts the effect of the delay due to the decrease of the area.

But if the hypothesis is justified at a certain moment, the simple equation which proceeds from it

$$S_T = S_0 e^{-KT}$$

must represent the phenomenon at the beginning and must express the part played by the first factor, the contraction, which intervenes alone at this moment, as long as the second disturbing factor does not enter into action, or its part is small with reference to that of the first one.

We can verify the correctness of this statement by drawing the curve representing the contraction of a wound; this can be done by measuring the total area of the new scar tissue, no longer merely the area of granulations. For we know that the decrease of this area measures solely the contraction.[3] Then the contraction curve obtained in this way should logically, within certain limits, comply with the law expressed by equation (3). Text-fig. 4 illustrates this fact, and our first hypothesis was therefore justifiable.

It is easily seen that the phenomenon follows the law until, owing to the decrease of the wound area, a more important part of the work of reparation in respect to the area of the granular surface is carried out by the second factor. Then, the decrease of the area being much greater than indicated by equation (3), the contraction, which depends obviously on the area not yet cicatrized, slackens gradually, until it ceases entirely. These observations would show plainly, if we were not already aware of it, that the second factor is the epithelization, and it is then understood that its action is represented in equation (9) by the quotient $\frac{T^2}{2p}$, which expresses that its efficiency, feeble at the beginning, increases slowly at first, then more rapidly, according to a parabolic law.

The above statements are generally verified only if the first observations are made when the cicatrization has already begun, and little or no epithelization has yet appeared. The starting-points of both curves (contraction and cicatrization) are confounded, that is they have the same ordinate at the time 0, so that the surface of the wound itself and that of the cicatrix cannot be discerned from each

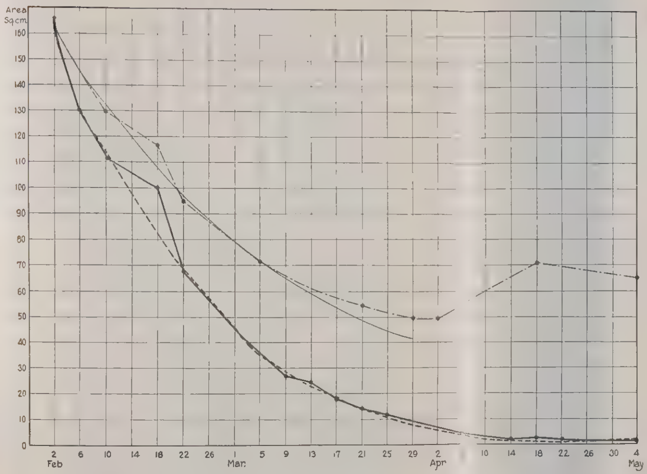
[3] Carrel, A., and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, 429.

other, the edges of the wound being constituted by the old skin or a new and hardly visible epithelial border (Text-fig. 4). When epidermization has already begun, the ordinates at the time 0 are not coincident. If S_o is the ordinate of the area of granulations, in square centimeters, and S_1 the area of the cicatrix, let us call A the difference $S_o - S_1$. A represents the surface already covered by the new epithelium and the equation becomes

$$S_1 = A + S_o c^{-KT}$$

$A + S_o$ is what we have called the area of the cicatrix. But in this case it is often more difficult to verify formula (3) for the contraction, because the epithelization may have become important enough to disturb the simple phenomenon of contraction, the disturbing action being obviously the function of A. The difficult definition of the outline of the cicatrix is also a cause of error. This explains why it is difficult, except on experimental wounds, to find cases on which observation can be made accurately. However, Text-fig. 5 shows that this is possible. The measure of the cicatrix area is made by drawing on cellophane the common limit of the old skin and of the new epithelium, or scar tissue. It is essential to draw this outline on the skin itself, in order to prevent errors of interpretation and of drawing which are frequent, as this common limit often lacks sharpness. But if at the beginning it is tattooed (on animals) or drawn with a dermographic pencil (on men), the measures become comparable and can be done with sufficient accuracy. Every time a drawing is taken, it is advisable to go over the outline again with the pencil where it shows a tendency to be obliterated.

As regards the term $\frac{T^2}{2p}$, what has already been said concerning its growing action in function of time must be taken merely from a mathematical standpoint and not as an assumption dealing with the mechanism of the phenomenon itself. The activities of the real factors are not known, and we can only measure one of the results of these activities, which may vary proportionally to the mathematical factors. Our knowledge does not go beyond that. For example, we know that $\frac{T^2}{2p}$ increases slowly at first, then rapidly, and we assume



TEXT-FIG. 4. Patient 360 The dotted and broken line represents the contraction of the wound (for details of technique see Carrel and Hartmann[3]). The light line is the calculated curve, according to the formula

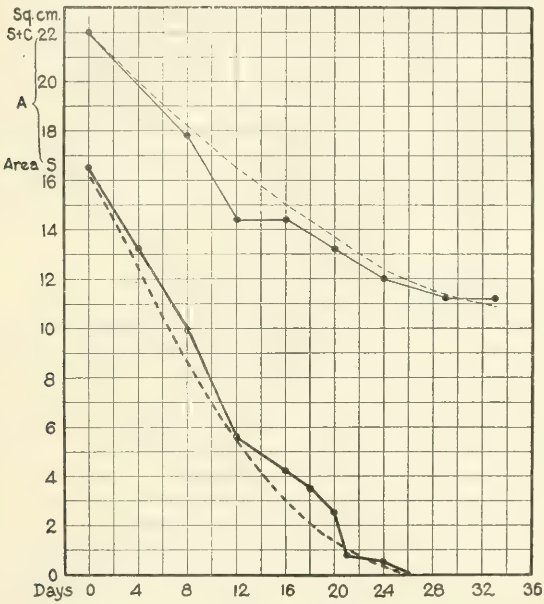
$$S_T = S_0 e^{-KT}$$

The heavy line represents the decrease of the area of the wound (curve of cicatrization) and the dotted line, the curve calculated according to the equation

$$S_1 = S_0 e^{-K\left(T + \frac{T^2}{2p}\right)}$$

The decrease in the rate on February 18 is due to infection

that this factor represents the epithelization. It must not be inferred that the latter remains proportional to $\frac{T^2}{2p}$ and increases at first slowly, then rapidly. On the contrary, we know that epitheliza-



TEXT-FIG. 5. The upper curves are the contraction curves. The dotted curve is calculated according to the formula

$$S_T = S_0 e^{-KT}$$

The lower curves are the so called cicatrization curves expressing the decrease of the area of the granulations.

tion, or growth of cells, is likely to be much more active at the beginning of the cicatrization, according to the length of the epithelial edge, and then must decrease in absolute value. In proportion as the

wound decreases, the length of the epithelial edge diminishes, and at the same time the number which measures, in absolute value, the proliferation of cells. But, as it is likely that the number of cells produced by a unit of length is the same for each unit of time, and as, on the other hand, ordinarily the area decreases much faster than the perimeter (four times more rapidly for the square), it is clear that the production of cells by the edges seems to increase and that if it is expressed by units of covered area it increases really with reference to the area of the wound. What must therefore be understood by "the factor represents the epithelization," is that in the considered equation owing to the introduction of this factor, the relations existing between epithelization and the decrease of the area are satisfactorily expressed, and that it enables us to express the result of the phenomena in a way which is in accordance with the facts.

End of the Phenomenon.

Since in a logarithmic curve the diminution of the ordinate is always proportional to the ordinate, it never becomes zero. The curve, as well as that which had been established previously, is asymptotic to the axis of the time. But we have already stated, in a former paper, the moment at which cicatrization comes practically to an end.[4] This happens when our methods of measuring are unable to estimate the progress of the phenomenon. This moment is rapidly followed--in a few hours—by complete healing of the wound. The curve practically comes to an end, and experience has shown that it can be arbitrarily stopped, when the ordinate is inferior to 0.4 sq. cm. This means that, when the calculation comes to a figure smaller than 0.4 sq. cm., the corresponding abscissa, that is the time, indicates the date of complete cicatrization. Besides, this conforms to the facts in the majority of cases, as has been shown before, and the errors are small. In all natural phenomena, the law of which is expressed by an exponential equation, the same holds true.

Numerical Value of the Coefficients. Relation of K, the Index of Cicatrization i, and the Parameter $2p$.

Calculation of fifteen cicatrization curves has shown principally three facts. The first, to which I referred above (page 334), is the pro-

[4] du Noüy, P. L., *J. Exp. Med.*, 1916, xxiv, 451; 1917, xxv, 721.

portional variations of K and of the index of cicatrization. Table II shows this plainly. The ratio $\frac{i}{K}$ varies between 1.6 and 1.2 inversely to i and K. The second fact is the remarkable constancy of the factor $2p$, or parameter of the parabolæ expressing the acceleration due to the epithelization. The third fact is the relation which seems

TABLE II.

Comparative Numerical Results.

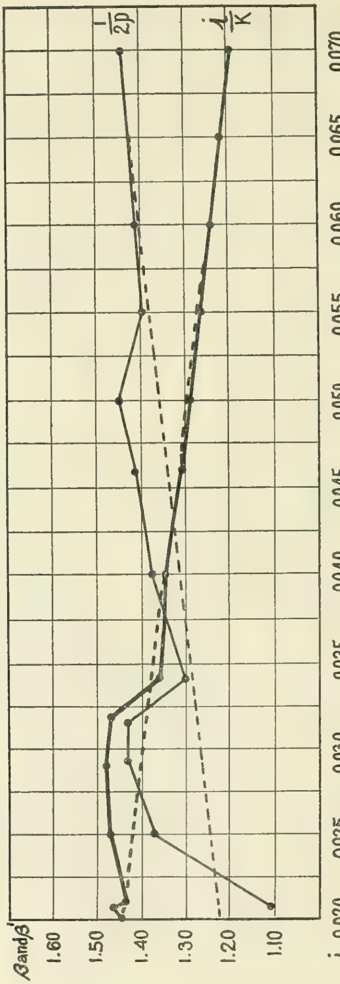
No. of patient.	Area.	Index i.	K	$2p$	$100\frac{K}{i} = \delta$		$\frac{i}{K} = \beta$
						$\frac{1}{\beta'}$	
	sq. cm.						
318	64.0	0.0200	0.0132	80	66		1.51
737	50.3	0.0200	0.0138	90	69	81.0	1.45
263	61.8	0.0205	0.0140	85	68	80.5	1.46
360	113.0	0.0210	0.0147	90	70	80.0	1.43
795	21.6	0.0255	0.0174	73	68.5	79.0	1.46
721	40.4	0.0285	0.0192	70	68	78.5	1.46
706	27.4	0.0325	0.0222	70	68.5	77.5	1.46
724	13.9	0.0346	0.0255	77	74	77.0	1.36
725	30.6	0.0375	0.0277	75	74	76.0	1.35
791	23.0	0.0400	0.0295	73	74	75.5	1.35
602	31.2	0.0420	0.0315	75	75	75.0	1.33
722	19.0	0.0465	0.0355	71	76	74.5	1.31
383	17.5	0.0500	0.0387	69	77	74.0	1.29
796	8.9	0.0550	0.0436	72	80	72.0	1.26
715	9.5	0.0700	0.0595	65	85	66.0	1.20

to exist between K, i, and $2p$. This is clearly shown by the seventh column in which is reported the term

$$\delta = 100\frac{K}{i}$$

The value of δ for each wound is near enough to $2p$ to allow its substitution for $2p$, approximately. This result is a natural conclusion of the first two remarks, since, if we call β the ratio $\frac{i}{K}$, we can write

$$2p = \frac{1}{\beta} = \frac{\delta}{100}$$



Text Fig. 6. Relations between i, K, and $2p$. The ordinates represent $\frac{i}{K} = \beta$ and $\frac{1}{2p} = \beta'$. Light lines, β'. Heavy lines, β.

But this is of immediate value for calculating the curve by equation (9), because, if the coefficient K can be determined by giving two experimental dates 4 days apart, the same process cannot be used for determining the parameter $2p$, for the parabola

$$\alpha = \frac{T^2}{2p}$$

can only be determined when T is great enough; *viz.*, 12 or 16 days. Otherwise the ordinates α are smaller than 1 and the curve is not accurately defined. It is therefore worth while to be able to make an approximate calculation first. If this shows a noticeable error, it is easy to make a correction, as soon as several days have passed. Consequently, it is clear from the above paragraphs that it is possible to calculate the curve resulting from the equation

$$S_T = S_0 e^{-K\left(T + \frac{T^2}{2p}\right)} \tag{9}$$

by simply starting from a single measure of the wound and the age of the patient, that is from the index, since

$$K = \frac{i}{\beta}$$

Text-fig. 6 shows the relations between K and i. In order to make it clearer, I have plotted close to each point on the observed curve (dotted line) the inverse value of β; that is, $\frac{1}{\beta}$. The light curve which expresses the observed variations of $\frac{1}{2p} = \beta'$ shows that $2p$ varies approximately inversely to β since every point on the curves can be expressed by the inverse value of the ordinate. This means that $2p$ varies inversely to δ. If we admit as a possibility that the light dotted line corresponds to the average mean value of $2p$ and that the values which deviate from it are due to errors of calculation, the probable values of $2p$ can be computed for a certain value of i. The figures in the column marked $\frac{1}{\beta'}$ (Table II) may be used for the first approximation of $2p$.

Calculation of the Coefficients.

These analogies may be of use in determining $2p$, but sometimes a less accurate approximation is obtained when both K and $2p$ are inferred from the index. In such a case the direct calculation of K, which is extremely simple, is of more value. It is deduced, as stated above, from equation (9) which gives

$$K = \frac{1}{T} \operatorname{Log} \frac{S_o}{S_t}$$

S_o being the first measure of the area of the wound, S_t the area at the time t (practically 4 days). After K has been determined, at least two values of α must be calculated unless the relations between the coefficients, mentioned above, are employed. We have stated

$$(7) \qquad \alpha = \frac{1}{K} \operatorname{Log} \frac{S_o}{S_T} - T$$

The values of S corresponding to 12 and 20 days, for instance, are taken. (The greater T is, the more accurate the values of $2p$ will be within the limits of 20 to 40 days.) $2p$ is immediately obtained by means of the formula

$$(8) \qquad 2p = \frac{T^2}{\alpha}$$

Since we have two values of α, we obtained two values of $2p$ and the mean value is taken.

The coefficient K is smaller than i and the quantity α must remain positive. If the contrary happens ($\alpha < 0$), a determination of K for a longer period of time (5, 6, or 8 days) must be made. This rarely happens.

Use of the Equation. Calculation of the Curves; Numerical Examples.

In order to enable the reader who is unfamiliar with the use of mathematical formulas, to use this equation, we shall make the complete calculation of one curve by using successively the direct calculation, or ordinary method, i being the supposed unknown, and then the method based upon the analogies existing between the coefficients. The difference in accuracy of both methods will thus be noted. Whenever the index i varies around 0.04 the results obtained by the second

technique are excellent. The reason is evident from Text-fig. 6 in which the values of the coefficients are in accordance with this particular value of i.

(*1*) *Direct Calculation.*—Only the initial area and that after 4, 8, or 20 days are given (Table III).

TABLE III.

Example of Direct Calculation.

T	S, observed area.	Log S	Log $\frac{S_o}{S}$	α	$2p$	Calculated area.
	sq. cm.					*sq. cm.*
0	40.4	1.602				
4	33.5	1.525	0.077	0		
8	27.0	1.431	0.171	0.9	71	27.0
20	12.5	1.097	0.505	6.2	65	12.8
28	6.8					7.0
36	3.5					3.5
44	1.7					1.7
54	0.6					0.6

The calculation requires accordingly (a) the determination of K

$$\left(K = \frac{\text{Log } S_o - \text{Log } S_T}{T}\right), \qquad K = \frac{0.077}{4} = 0.0192$$

(b) the determination of α (8th day)

$$\left(\alpha = \frac{\text{Log } S_o - \text{Log } S_T}{K} - T\right), \qquad \alpha = \frac{0.171}{0.0192} - 8 = 0.9$$

(c) the determination of $2p$ (8th and 20th days)

$$\left(2p = \frac{T^2}{\alpha}\right), \qquad 2p = \frac{64}{0.9} = 71$$

and finally the calculation of the points of the curve for the given times by formula (9)

$$\text{Log } S_T = \text{Log } S_o - K\left(T - \frac{T^2}{2p}\right)$$

(*2*) *Indirect Calculation.*—This is based only upon the relations previously stated between i, K, and $2p$. $2p$ may be taken either from

Table II or from Text-fig. 6, for the given value of i. In the preceding example $i = 0.0285$ (Table V). For this value, the table indicates $\frac{1}{\beta'} = 2p = 78.5$. The factor given by Text-fig. 6 is 1.41. Hence $K = \frac{i}{1.41} = 0.0202$. By applying the formula the areas given in Table IV are calculated (compare with Table III).

This shows that the values are as good as those obtained by the direct method, sometimes even better, because in the latter method $2p$ has only been determined from two points on the curve, which is a cause of error.

A direct determination of K can also be made and only the value of $2p$ read in the table. The results obtained by this intermediate technique are good, but it shows no particular advantage, and, on the contrary, introduces a new factor of error and requires more time.

TABLE IV.

Area (S).					
8th day.	20th day.	28th day.	36th day.	44th day.	54th day.
sq. cm.	*sq. cm.*	*sq. cm.*	*sq. cm.*	*sq. cm.*	*sq. cm.*
27.0	12.4	6.8	3.5	1.6	0.5

It must be borne in mind that the determination of a curve by a single measure of the area and the normal index presents many advantages which may be of greater interest than the perfect coincidence between two curves obtained by two equations of different form. The advantages are: (1) the possession of the normal curve of cicatrization corresponding to the normal index, characterizing the age of the patient; this curve is used as a standard with which the individual curve is compared, if they do not agree; (2) the elimination of errors due to two measures of the wound, because during the time elapsed between the measures—4 days for example—a slight acceleration or a slight lessening in the rate might have occurred. The so called indirect method, therefore, should be used when the normal curve of a wound is to be calculated. In order to facilitate this calculation, I have drawn Text-fig. 7 similar to Text-fig. 6, except that the ob-

TABLE V.

Calculation of the Curve of Cicatrization.

The Two Coefficients of the Formula

$S' = S\left[1 - i\,(t + \sqrt{nt})\right].$

Area of wound.	1st coefficient—index of cicatrization i.					2nd coefficient (time coefficient) $t + \sqrt{nt}$.
	Age of patient.					
	20 yrs.	25 yrs.	30 yrs.	32 yrs.	40 yrs.	
sq. cm.						
150 and over.	0.0200	0.0200	0.0200	0.0200	0.0200	6.00
						6.81
						7.43
140	0.0210	0.0200	0.0200	0.0200	0.0200	8.00
						8.45
130	0.0220	0.0200	0.0200	0.0200	0.0200	8.90
						9.30
120	0.0225	0.0200	0.0200	0.0200	0.0200	9.65
						10.00
110	0.0240	0.0200	0.0200	0.0200	0.0200	10.32
						10.64
100	0.0250	0.0200	0.0200	0.0200	0.0200	10.93
						11.21
90	0.0275	0.0220	0.0200	0.0200	0.0200	11.48
						11.75
80	0.0300	0.0230	0.0200	0.0200	0.0200	12.00
						12.25
70	0.0325	0.0250	0.0200	0.0200	0.0200	12.48
						12.72
60	0.0355	0.0300	0.0225	0.0200	0.0200	12.95
						13.16
50	0.0400	0.0340	0.0265	0.0230	0.0200	13.37
						13.60
40	0.0445	0.0400	0.0310	0.0270	0.0220	
30	0.0500	0.0450	0.0375	0.0330	0.0260	
25	0.0540	0.0500	0.0400	0.0375	0.0290	
20	0.0580	0.0540	0.0465	0.0425	0.0325	
15	0.0645	0.0600	0.0525	0.0475	0.0380	
10	0.0700	0.0660	0.0625	0.0550	0.0450	
5 and under.	0.0800	0.0750	0.0700	0.0700	0.0700	

served points are suppressed and the scale of the ordinates is larger, so that a greater accuracy may be obtained. To show the degree of approximation obtained by the new technique I have collected the calculations of four wounds. The figures calculated according to the extrapolation and exponential equations correspond to every observed area. These curves have been chosen intentionally, so that their indices are different. The calculation of the coefficients K and $2p$ is then simply done by looking for the index in Table V in function of the age of the man and of the area of the wound; then by using the relations (Table II)

$$K = \frac{i}{\beta} \text{ and } 2p = \frac{100}{\beta'}$$

calling β the observed values of $\frac{i}{K}$ (solid line) and β' the observed values of $\frac{1}{2p}$ (dotted and broken line), the values of $2p$ can also be found in Text-fig. 7, since the values of Table II have been calculated from this straight line. Text-fig. 7 is only used in order to give two conversion factors β and β', to be applied for computing K and $2p$.

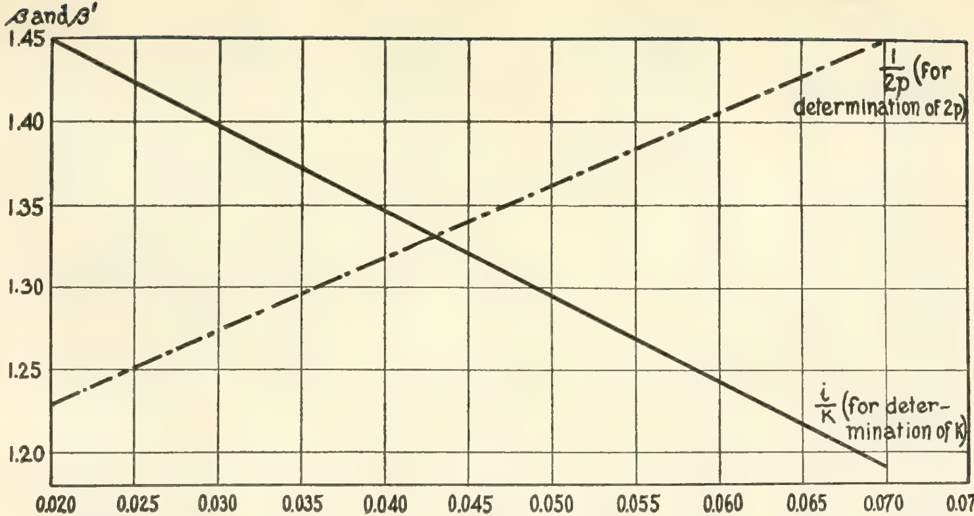
Comparative Examples.

Patient 360.

$$S_o = 129.4 \text{ sq. cm.}, i = 0.021, \beta = 1.44, \beta' = 1.23, K = \frac{i}{\beta} = 0.01458,$$

$$2p = \frac{1}{\beta'} = \frac{1}{1.23} \times 100 = 81.$$

Area.	8th day.	20th day.	44th day.	60th day.	76th day.	84th day.
	sq. cm.	*sq. cm.*	*sq. cm.*	*sq. cm.*	*sq. cm.*	*sq. cm.*
Observed	105.0	57.0	13.8	4.1	1.8	0.6
Calculated Equation (1)	96.8	55.9	14.0	4.7	1.9	1.0
Calculated " (9)	96.5	56.0	13.4	3.9	1.4	0.4



TEXT-FIG. 7. Relations between i, K, and $2p$. The chart gives the ratio $\beta = \frac{i}{K}$ and $\frac{1}{2p} = \beta'$, by means of which K and $2p$ can be calculated.

Patient 488.

S_o = 34.5 sq. cm., i = 0.03, β = 1.40, β' = 1.27, K = 0.0214, $2p$ = 79.

Area.	8th day.	20th day.	32nd day.	44th day.	48th day.
	sq. cm.	*sq. cm.*	*sq. cm.*	*sq. cm.*	*sq. cm.*
Observed	20.6	11.0	4.0	1.7	0.9
Calculated { Equation (1)	22.4	9.9	3.7	1.3	0.8
Calculated { " (9)	22.2	10.0	3.8	1.2	0.7

Patient 694.

S_o = 44.3 sq. cm., i = 0.0425, β = 1.33, β' = 1.33.

Area.	8th day.	16th day.	20th day.	28th day.
	s .	*sq. cm.*	*sq. cm.*	*sq. cm.*
Observed	23.6	11.2	8.5	2.5
Calculated { Equation (1)	23.5	10.5	6.9	2.4
Calculated { " (9)	23.1	10.7	6.8	2.6

Patient 519.

S_o = 19.0 sq. cm., i = 0.0570, β = 1.26, β' = 1.39.

Area.	4th day.	12th day.	20th day.	28th day.
	sq. cm.	*sq. cm.*	*sq. cm.*	*sq. cm.*
Observed	12.2	4.2	1.0	0.4
Calculated { Equation (1)	12.5	4.4	1.2	0.3
Calculated { " (9)	12.2	4.4	1.3	0.3

CONCLUSION.

1. The law of cicatrization of surface wounds may be expressed by an exponential formula with two coefficients which may be easily determined.

2. A simple relation exists between these coefficients and the index, i, of cicatrization, previously established in function of the age of the patient and of the area of the wound.

3. The proposed equation with a simplified exponent, reduced to a single coefficient, expresses satisfactorily the phenomenon of contraction.

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THE PHYSIOLOGICAL BASIS OF MORPHOLOGICAL POLARITY IN REGENERATION. I.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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INTRODUCTION.

In two preceding papers[1] on regeneration in *Bryophyllum calycinum* it had been shown that the mass of shoots formed in a leaf or in a piece of stem to the base of which a leaf is attached increases with the mass of the leaf. This proves that regeneration is a phenomenon of the order of nutrition and growth.

The question arises how this conclusion harmonizes with the well known fact of the polar character of regeneration. When we cut out a piece from a higher plant or a lower animal the piece regenerates, as a rule, a shoot or head at the apical end and roots or a tail at the basal end. In a preliminary paper,[2] published a year ago, the writer described experiments suggesting that the polar character of regeneration might be due to the existence in the circulating sap (or lymph and blood) of inhibitory substances which prevent dormant buds or resting cells from growing out even if an adequate quantity of food-stuffs is available. It was shown by experiments on *Bryophyllum calycinum* that the leaves as well as the growing shoots have an inhibitory influence upon the growth of all the dormant buds situated more basally in the stem. If we assume that these inhibitory influences are due to certain constituents in the sap sent out by growing buds and by leaves, we come to the following theory of the polar character of regeneration. When we cut out a piece of stem from *Bryophyllum* and remove all the leaves, inhibitory substances will continue to flow in a basal direction in the stem. Since the apical

[1] Loeb, J., *Bot. Gaz.*, 1918, lxv, 150; *J. Gen. Physiol.*, 1918, i, 81.

[2] Loeb, *Science*, 1917, xlvi, 547.

region of the piece will be the first to become sufficiently free from these substances, the buds situated at this end will be the first to grow out into shoots. As soon as this happens the new shoots will in their turn send out inhibitory substances in a basal direction in the stem, thereby preventing the growth of the more basally situated buds. In this paper we shall present some of the qualitative evidence for the inhibitory effect of a leaf upon shoot formation, leaving the discussion of experiments of a more quantitative character for a future paper.

FIG. 1. Diagram showing arrangement of shoot buds in the stem of *Bryophyllum calycinum*. The line connecting the two buds in one node is always at right angles to the line connecting two buds in the next node.

We shall deal chiefly with the regeneration of shoots in the stem of *Bryophyllum calycinum*, which can proceed only from definitely located buds. In the axil of each leaf of a stem there exists one bud capable of giving rise to a shoot, which, however, never does so unless the plant is mutilated. Each node of a plant has two leaves in opposite position, and the axis connecting the two axillary buds in one node is always at right angles with the axis connecting the two buds of the next node (Fig. 1). Thus the line connecting the two buds at Node 2 (Fig. 1) is at right angles with the line connecting the two buds in Nodes 1 and 3, etc. The lower leaves in a stem fall off in time, leaving their axillary buds exposed. No other element of the stem except the two buds in each node is capable of growing into shoots

The elements capable of giving rise to roots are not confined to the nodes but exist all over the stem in a definite layer of the cortex. It can be shown that the sap from the leaf flowing towards the base of a stem favors the growth of roots and inhibits the growth of shoots.

Experiments on Potted Plants.

When we cut off the top of a potted plant of *Bryophyllum calycinum*, leaving a stem containing only two leaves at the apical node, none

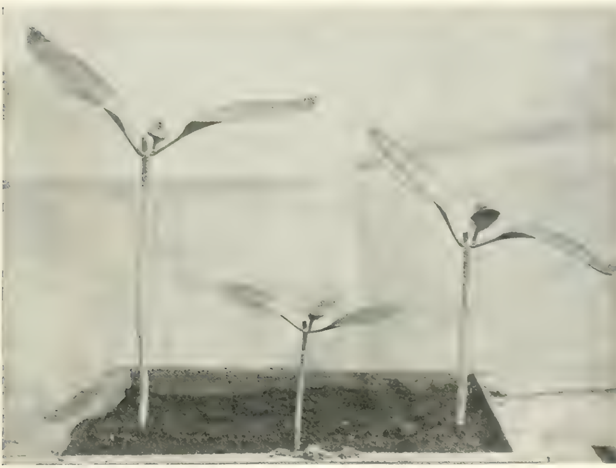

FIG. 2. Top of plant removed; two leaves at apex which prevent growth of all the buds below.

of the buds on the stem below the two leaves will grow out (Fig. 2), so long as the leaves are alive and able to send their sap to the base of the plant. The buds situated in the axil of the two leaves may after some time grow out. The two leaves inhibit therefore the growth of all the buds situated more basally (Fig. 2). Each leaf inhibits the growth of the buds situated in the same half of the stem, and in order to prove this we remove in a second set of experiments

the top of a number of potted plants leaving only one leaf at the apex (Fig. 3). We must also remove the free bud opposite this leaf, since otherwise this bud will grow out and produce the same inhibitory effect as the removed leaf would have done. In this case the experiment would only be a repetition of the preceding experiment in which two leaves were left at the apex. When, however, we remove one apical leaf with its axillary bud very often the one leaf left at the top suffices to suppress regeneration in the basal part of the stem

Fig. 3. One leaf left at apex. Growth of shoots in the first node below the leaf suppressed, while the shoot in the second node below the leaf, but on the opposite side of the stem can grow out.

as completely as if two leaves had been left. If regeneration occurs, it takes place in the highest node on the opposite half of the stem which is the second node below the leaf (Fig. 3). In the first node below the leaf no bud can grow out. I have never noticed an exception to this rule in a normal plant. A possible explanation of this phenomenon is furnished by Fig. 4, where that half of the stem through which the sap from the apical leaf flows to the base of the stem is shaded. Since the buds in the first node below

the apical leaf are in the path of the sap flow from the leaf, the formation of shoots is suppressed in these buds, while the bud in the second and fourth nodes below but on the opposite side from the leaf lies outside of the path of the conducting vessels from the leaf. Hence if any bud in such a stem grows out it is usually the one in the second node below but on the opposite side from the apical leaf. As soon as this bud grows out it will inhibit the growth of the lower buds in the same half of the stem.

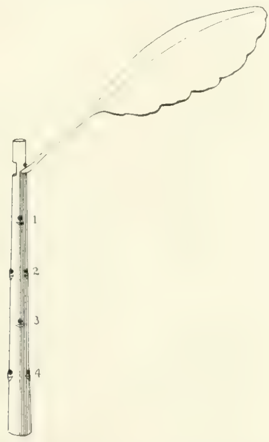

FIG. 4. Diagram explaining this inhibitory influence of the leaf on the theory that the descending sap from the leaf carries inhibitory substances. The region of the stem reached by the sap from the leaf is shaded and in the shaded part regeneration of shoots is inhibited.

The petiole of a leaf is attached with its base to one-half of the circumference of the stem. When we cut off half of the base of the petiole of a leaf, the sap sent out by that leaf can flow only through one quadrant of the next internode. This should limit the inhibitory influence of such a leaf to this quadrant of the node below, and this turns out to be the case. The top of a number of potted plants was cut off and only one leaf was left at the apex (Fig. 5). Half of the

FIG. 5. One-half leaf and one-half petiole left at apex. One of the two shoots in node below leaf now grows out; namely, on the side where half the petiole is removed.

petiole of this leaf was removed at the base and also the corresponding half of the leaf itself was cut off, though this latter procedure is not essential for the result. The axillary bud of the other leaf was also cut out as in the preceding experiment. Fig. 5 gives the result of such an experiment. The reader will notice that in this case one of the two buds in the first node below the leaf will grow out; namely, that one which lies beneath the removed half of the leaf. This bud

FIG. 6. Showing that the inhibitory influence of an apical leaf upon shoot formation in the node below the leaf disappears when the size of the leaf is sufficiently diminished. Duration of experiment, Oct. 25 to Nov. 14.

grows out since it no longer receives any of the sap from the leaf above. I have never seen the other bud in this node grow out. This experiment also succeeds in practically every case.

We have seen that if we remove one leaf and its axillary bud at the apex of a topped stem, leaving only one leaf at the apex, the buds in the node below are practically always prevented from growing out. When we diminish the mass of the leaf sufficiently (as is done in Fig. 6), this inhibitory influence ceases and every plant forms a shoot in

FIG. 7. Control experiment to Fig. 6. Duration of experiment also from Oct. 25 to Nov. 14.

each of the two buds in the first node. Fig. 7 shows the control experiment; namely, six stems each with one whole leaf at the apex. Not a single stem has formed a shoot in the first or any other node below the leaf. Both sets of experiments were carried out simultaneously and both sets of plants were side by side in the same flower bed.

When we reduce the mass of a leaf 10 days after the experiment is started and when the new shoots begin to form, the inhibitory effect nevertheless becomes noticeable.

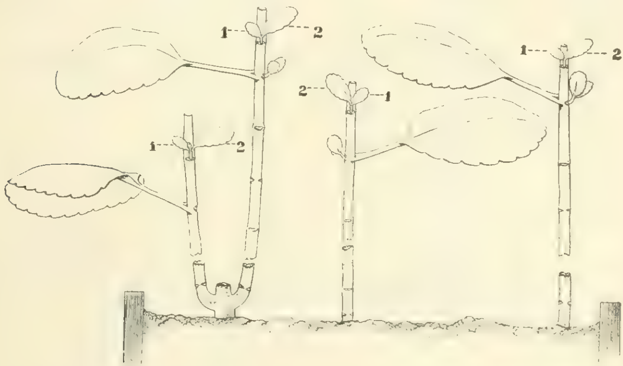

FIG. 8. Proof that traces of inhibition of a leaf upon shoot formation are also noticeable in the more apical shoots. Old leaf left at first node below the apical node. The two Leaves 1 and 2 of the new shoot in the apical node which are normally of equal size show a constant difference, Leaf 1 (on the side where the old leaf is) being smaller than Leaf 2 (on the opposite side).

Demonstration of the Inhibitory Influence of a Leaf on Shoot Formation in a More Apical Node.

We can state as a general rule that a leaf accelerates the growth of shoots at the apex and prevents or retards it in the basal parts of the stem. The leaf has, however, also a slight inhibitory effect on the more apical buds. In order to prove this it is necessary to make experiments like those represented in Fig. 8. In a number of topped

plants one leaf is left at the node below the most apical one. In this case both buds in the most apical node grow out into a shoot giving rise as usual to two small Leaves 1 and 2. While these leaves are normally of equal size, a typical and constant difference exists between the size of the two leaves when one old leaf is left in the node below. Leaflet 1, which has the same orientation as this old and large leaf in

FIG. 9. The inhibitory influence of the leaf upon an apical bud disappears also when the size of the leaf is reduced. Duration of experiment, Oct. 24 to Nov. 7.

the node below, is practically always smaller than the other, Leaflet 2 (Fig. 8). This difference is intelligible on the assumption that a small quantity of the inhibitory substances from a leaf flows towards the apex of the stem; these substances will reach the young leaf facing the same side of the stem where the old leaf is, while they do not reach the other leaf. When we reduce the size of the old leaf, this inhibitory influence disappears (Fig. 9).

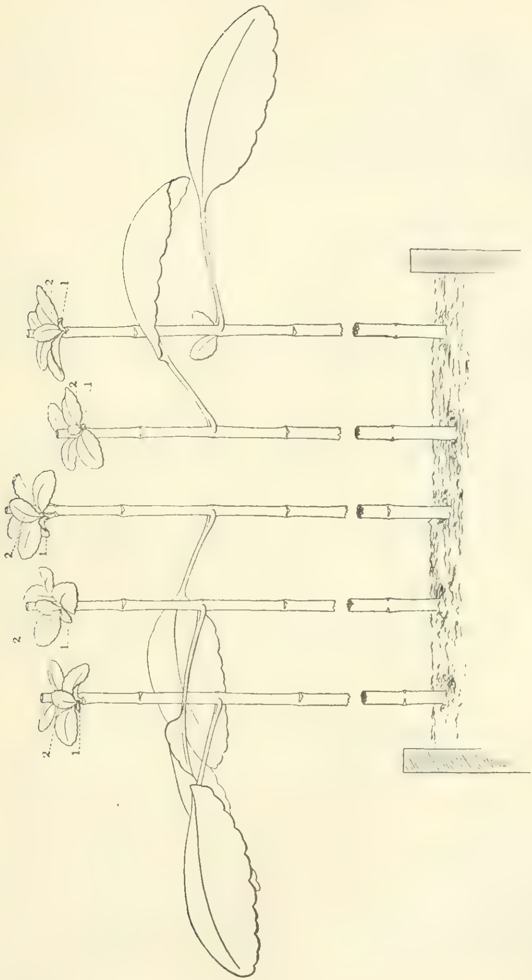

Fig. 10. In the second node above the old leaf the inhibitory effect is shown in the second pair of leaves of the new shoot which are no longer of equal size, Leaf 1 which is above the old leaf being a little smaller than the symmetrical Leaf 2. Duration of experiment, Oct. 17 to Nov. 13.

This slight inhibitory influence of a leaf upon the more apical buds shown in Fig. 8 can also be demonstrated in the growth of the second node above a leaf (Fig. 10). In this case the influence is noticeable only in the second pair of new leaves of a bud; Leaf 1 which has the same orientation as the old leaf remains smaller than Leaf 2.

While in the basal region of a leaf the inhibitory effect is complete, it is comparatively slight in the apical part.

Influence of Gravity upon the Inhibitory Action of the Leaf.

All the experiments on potted plants described in the preceding pages can be repeated with the same result in stems cut out from a plant. We may omit a description of such experiments since they would constitute only a repetition of what has already been stated. But certain of these experiments yield some additional results which are of theoretical importance.

The assumption that the inhibitory effect of the leaf upon the growth of dormant shoot buds is due to chemical substances sent out by the leaf is supported by the striking influence of gravity on regeneration in stems suspended horizontally. Long straight stems were cut out from a plant and suspended horizontally in an aquarium nearly saturated with water vapor. When two leaves are left at the most apical node of such pieces, none of the buds situated more basally will grow out. If, however, one leaf with its axillary bud is removed and the other leaf left, regeneration will occur, but the buds which will grow out will show a characteristic difference according to whether the leaf is on the upper or the lower side of the horizontally suspended stem.

We suspend such stems so that the axis of the two most apical buds (one of which is removed with its leaf) is vertical (Fig. 11). In five stems the leaf is on the upper side and in five stems on the lower side of the stem (Fig. 11). All the stems were originally horizontal but underwent the geotropic bending described in previous papers, whereby the upper side became concave.

When the leaf is below (right half of Fig. 11), shoots may be formed in the first node basally from the leaf. This occurred in three out of five stems drawn here. The other two formed shoots from the upper bud of the second node.

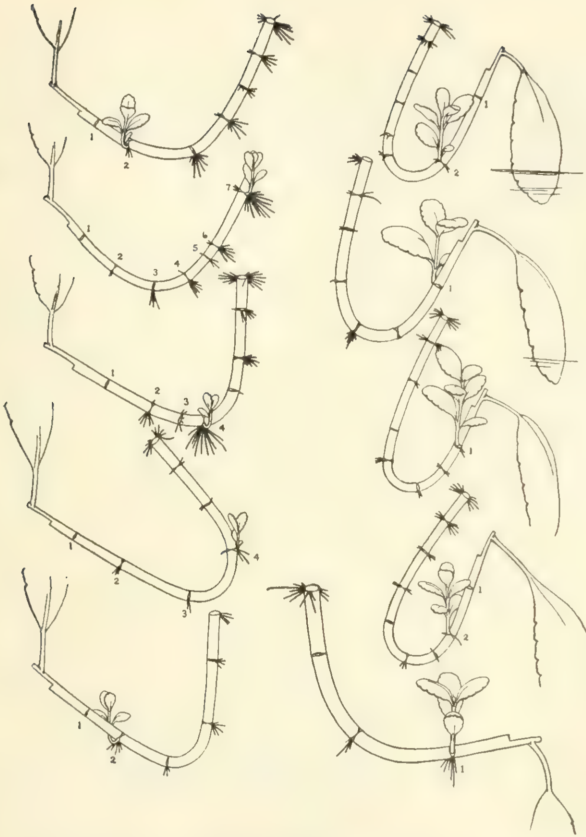

FIG. 11. Influence of gravity on shoot formation in horizontally suspended stems. The stems were originally straight but underwent geotropic bending during the experiment. In the stems to the right one leaf is left at the lower side of apex. In this case the shoots in the first node will grow out in half of the stems; in the second node only the upper bud will grow out. In the stems to the left the leaf is on the upper side. In this case the shoots in the first node are prevented from growing, and only the lower bud in the second and fourth node can grow out. Duration of experiment, Sept. 29 to Nov. 12.

When the leaf is above (left side of Fig. 11) none of the buds in the first node will grow out. If any buds grow out, they are either the second or fourth on the *lower* side of the stem. These shoots grow out with much delay compared with the growth of shoots in stems where the apical leaf is on the lower side of the stem.

The inhibitory influence is therefore greater when the apical leaf is on the upper than when it is on the lower side of a horizontally suspended stem. This influence of gravity supports the idea that it is the sap sent out by the leaf which produces the inhibition The diagrammatic Figs. 12 and 13 make this clear. In Fig. 12 the leaf is below and the path of the conducting vessels from the leaf is marked by black lines. The two buds of the first node lie on the upper edge of the sap flow containing the hypothetical inhibitory substances The buds of the first node may or may not receive enough of these substances to prevent their growth. When, however, the leaf is above (Fig. 13) seepage from the vessels will cause the buds in the first and third nodes to be flooded with the sap and the inhibitory substances contained in it, thus preventing their growth. The lower bud in Node 2 (or Node 4) is outside the direct path of the conducting vessels of the leaf and hence the lower bud of Node 2 as well as of Node 4 may develop. Through the influence of gravity traces of the sap may possibly reach the lower bud of the second or fourth node. This may account for the fact that growth of these buds is usually retarded.

The reader will notice that these facts give us a neat method of restricting the growth of shoots to the lower side of a horizontally suspended stem, contrary to the general rule that in such cases shoots arise on the upper side of the stem. When we remove the lower half of such a horizontally suspended stem (containing one leaf at the upper side of its apex) leaving on the lower side only the region of the second node (Fig. 14), regeneration of a shoot will occur only from the bud on the under side of this second node. The growth of the buds in the intact upper half of the stem is completely suppressed and the growth of the bud on the under side of Node 2 is slow for reasons stated. No growth will occur on the upper side, except after the leaf is wilted or conduction of its sap through the stem is interrupted.

The correctness of this idea is supported by the further fact that this inhibitory effect of a leaf on the growth of shoots, especially in the basal parts of the stem, is diminished when the mass of the leaf is reduced. Fig. 15 shows such an experiment. Of the five stems on the left each had one whole leaf on the upper side of the originally horizontal stem. In one stem only did a shoot form and this shoot

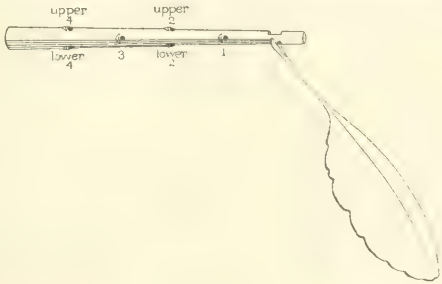

FIG 12.

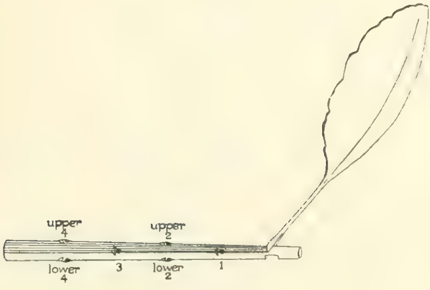

FIG. 13.

FIGS. 12 and 13. Explanation of the influence of gravity on regeneration in horizontally suspended stems on the assumption that inhibition is due to substances carried by the sap sent out by the leaf. When the leaf is below (Fig. 12), the buds in Nodes 1 and 3 are at the upper edge of the sap flow and these buds may or may not escape the inhibitory effect. In Fig. 13 the leaf is above and the sap flowing in the upper half is bound to reach the buds in Nodes 1 and 3 and hence their growth is necessarily suppressed. The lower Buds 2 and 4 are outside the sap flow and may develop.

developed on the lower side in the second node. In the five stems on the right the mass of the leaf was reduced considerably. Four of the five stems formed shoots, two even in the second node on the upper side of the stem. In a repetition of this experiment half of the stems with a reduced apical leaf on the upper side formed shoots in the first node basally from the leaf.

FIG. 14. Horizontally suspended stems. Leaf on upper side. The lower half of stem is removed with exception of second node. The lower bud of the second node grows out into a shoot, while the shoot formation on the upper side of the stem is inhibited by the leaf. Duration of experiment, Feb. 13 to June 24.

The reader will notice that the geotropic bending of the stems was considerably less in the five stems on the right with the reduced leaf than in the five stems on the left with a whole leaf.[3]

[3] Loeb, *Science*, 1917, xlvi, 115; *Ann. Inst. Pasteur*, 1918, xxxii, 1; Forced movements, tropisms, and animal conduct, Monographs on Experimental Biology, Philadelphia, 1918.

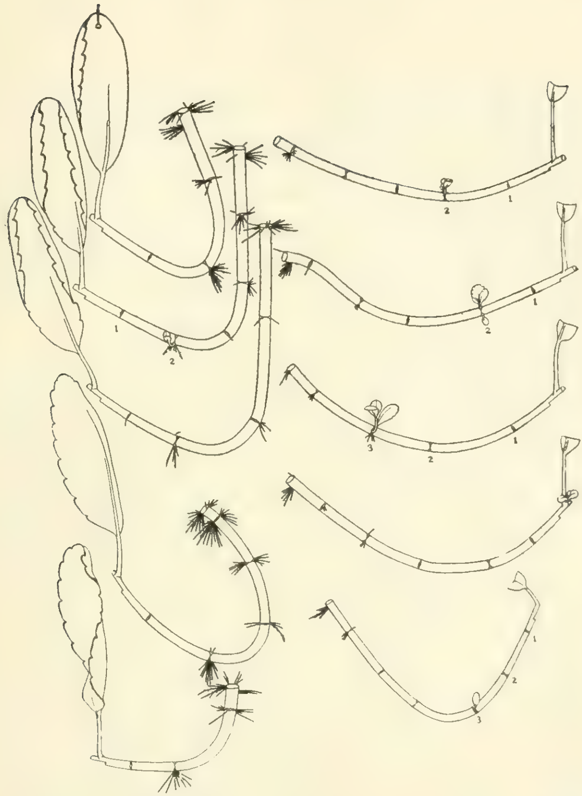

FIG. 15. Showing that reduction in size of apical leaf diminishes its inhibitory power. On the left, five stems each with large size leaf at apex, on upper side of stem. Inhibition of shoot formation complete except in one stem where a shoot is formed in the lower bud of the second node. On the right, five stems each with reduced leaf on the upper side. Four out of five stems form shoots and two of these do so in the upper bud of the second node. Root formation and geotropic curvature are considerably larger in stems with whole leaf than in stems with reduced leaf. Duration of experiment, Oct. 29 to Nov. 23.

Correlation between Inhibitory Effect of a Leaf on Shoot Production and the Opposite Effect on Root Production in a Stem.

The writer has already called attention to the correlation existing between the inhibitory effect of a leaf upon shoot formation and the opposite effect on root formation. This is expressed among others in Fig. 15. The stems on the left side, with a full size leaf at the apex, formed a considerably larger mass of roots in the same time than the stems on the right whose leaves are reduced in size. The larger the apical leaf the greater the mass of roots produced by the basal part of the stem in the same time and under equal conditions; and the greater the inhibitory effect upon the shoot production in this part of the stem.

A striking demonstration of this correlation is given in the upper row of drawings in Fig. 16. One-half of the leaf and one-half of its petiole were cut off. The leaf was at the base of the stem which contained only one node in front of the leaf. Generally only one of the two buds in the node situated apically from the leaf grew into a shoot; namely, the one on that side where half of the leaf was removed. The growth of the bud on the side where the half leaf was preserved was retarded or suppressed. At the basal end of the stems roots developed, but at first only on that side of the stem where the leaf was preserved.[4] Hence the leaf behaves as if it sent out, in addition to the material needed for regeneration, substances retarding shoot formation and favoring root formation.

In the lower row of stems (Fig. 16) the leaf was preserved at the apical end of the stem. In this case the inhibitory effect of the half leaf on shoot formation is much greater than when it is at the base of the stem (upper row). Most of the stems in the lower row have not yet formed any shoots, but where a shoot was formed (as in III*a*) it was formed on the opposite side from that where the half leaf was preserved, while the roots were on the same side with the leaf.

In Fig. 16 the half leaf was above. In Fig. 17 the half leaf was always below. The result in Fig. 17 is the same as in Fig. 16, *i.e.*, on the side where the half leaf is preserved the shoot formation is always retarded compared with that on the other side. The inhibition is more complete when the leaf is at the apex (lower row) than when the leaf is at the base (upper row).

[4] Later on roots may form on both sides of the basal end of the stem.

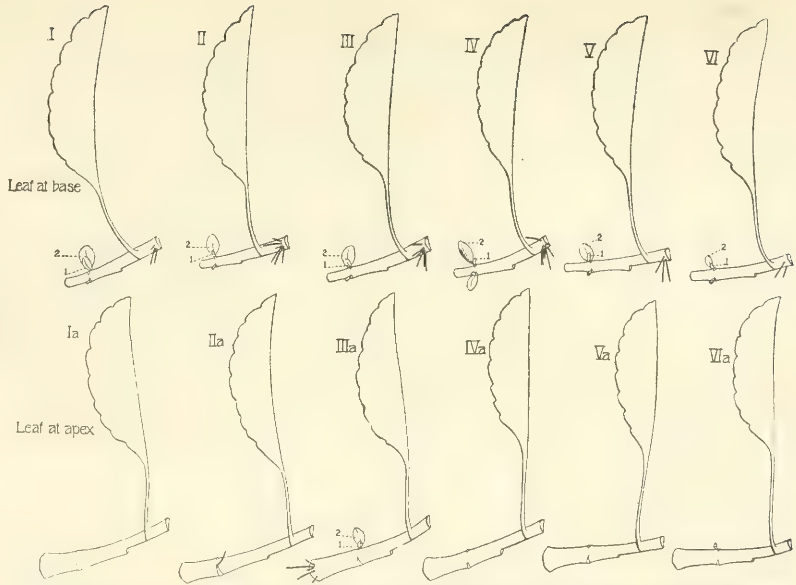

FIG. 16. Upper row: half leaf at base of stem. Shoot formation inhibited and root formation favored on that side of stem where petiole of leaf is preserved. Lower row: half leaf at apex. Inhibitory effect of leaf on shoot formation is more complete than in upper row. Duration of experiment, Oct. 24 to Nov. 9.

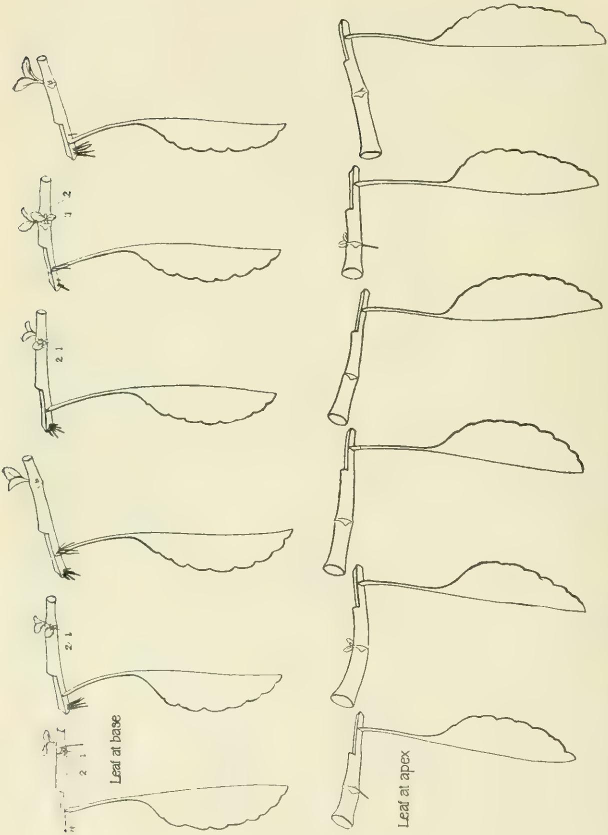

FIG. 17. Half leaf on lower side of stem. Similar result as in Fig. 16. Duration of experiment, Oct. 19 to Nov. 1.

Proof That the Leaf Sends Nutritive Material Also in the Basal Direction of the Stem.

When we suspend stems horizontally with one leaf at the apex, the inhibitory effect of the leaf upon shoot formation is much stronger when the apical leaf is on the upper side of the stem than when the leaf is on the lower side of the stem. We can use experiments of the latter type to show that the leaf sends nutritive substances to the base as well as to the apex and that the fact that the leaf inhibits shoot formation at the base is not due to the leaf failing to send nutritive material in the direction of the base of the stem. The method of proving this consists in measuring the influence of the mass of the leaf upon shoot formation in the basal part of a horizontally suspended stem.

Stems were split longitudinally and suspended horizontally; each stem having a leaf at the apex, and on the lower side of the stem.[5] Fig. 18 gives the result of such an experiment. Pieces of stems possessing two nodes and two leaves at the apical node are split longitudinally, so that each half stem has one leaf at the apex and one bud in the basal node. One leaf is left intact while the size of the sister leaf is reduced considerably. In Fig. 18 Leaves I and I*a*, II and II*a*, etc., are sister leaves. Practically each stem has produced a shoot at the basal node, but the shoot is invariably greater in the stems in the upper row where a whole leaf was at the apex than in the lower row where the apical leaf was reduced in size. The drawing was made on the 34th day of the experiment. It is obvious that the growth of the basal shoots increases with the mass of the apical leaves and this is proved by the relative weight of the leaves and shoots.

Duration of Experiment, 39 Days.

	Wt. of 4 whole leaves.	Mass of 4 shoots.	Shoots per gm. of leaf.
	gm.	*gm.*	*mg.*
Fresh.	17.035	2.128	125
Dry.	1.196	0.193	160
	Wt. of 4 reduced sister leaves.		
Fresh.	2.182	0.384	175
Dry.	0.140	0.027	193

[5] If the leaf is on the upper side, we get too much inhibition of shoot formation, whereby the experiment is rendered difficult.

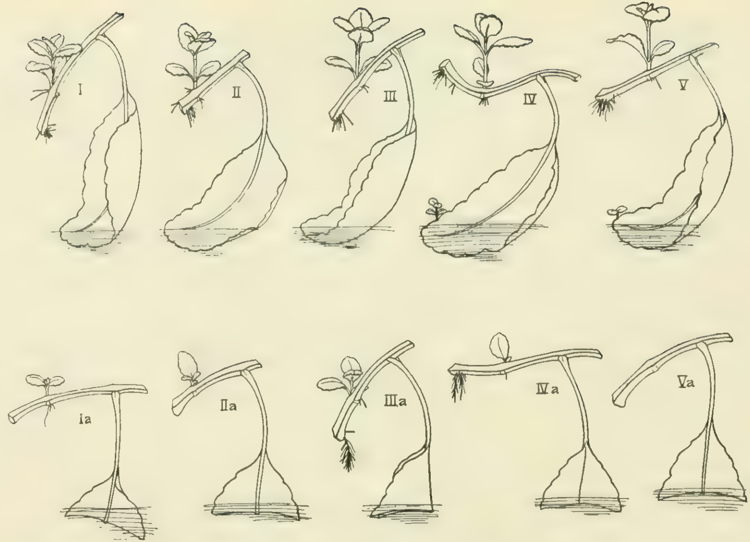

FIG. 18. Stems split longitudinally and suspended horizontally, with apical leaf on the lower side. Leaves I and I*a*, II and II*a*, etc., are sister leaves. The leaf in the upper row is intact, while in the lower row it is reduced in size. The mass of shoots produced is smaller in the stems of the lower row where the size of the leaf is reduced than in the upper row where the leaf is larger. This proves that inhibitory influence of leaf upon basal parts of stem is not due to lack of nutritive material.

Other experiments gave similar results. While the experiment does not reveal any strict proportionality between mass of leaf and mass of basal shoot produced, the increase of the mass of shoots with the mass of the leaves is unmistakable.

We therefore come to the following conclusion. The leaf sends material for growth in both directions of the stem, to the apex as well as to the base. It also behaves as if it sent out inhibitory substances in both directions, but if this be the case there must exist a considerable difference in regard to the mass of the latter. While much or almost all of the inhibitory substance is sent in basal direction, only traces of it are sent in an apical direction, so that special experiments are required to demonstrate the inhibitory effect in the apical parts of the stem.

A glance at Fig. 18 confirms also the statement that the mass of roots formed in the basal part of a stem increases with the mass of the apical leaf. The stems in the upper row with a whole leaf at the apex have a greater mass of shoots and roots than the stems in the lower row with a reduced leaf.

DISCUSSION.

We have shown in this paper that a leaf inhibits the regeneration of shoots in the basal parts of the stem and that this inhibition is diminished or ceases when the mass of the leaf is reduced below a certain limit. If the inhibitory influence of the leaf is due to inhibitory substances sent out by the leaf to the basal parts of the stem we must conclude that traces of these inhibitory substances flow also to the apex of the leaf since it is possible to demonstrate slight inhibitory influences of the leaf in the buds situated apically.

The influence of the leaf upon the regeneration of roots is exactly the reverse from that on the regeneration of shoots. The leaf favors the formation of roots in the basal parts of the stem and this favorable influence upon regeneration of roots in the basal part of a stem increases with the mass of the apical leaf.

This gives us an indication of the rôle which a leaf plays in the establishment of the polar character of regeneration in the stem of *Bryophyllum calycinum*. When a piece of stem is cut out with a leaf in the middle, the leaf sends out nutritive material in both directions of

the stem, since it can be shown that if once a shoot is caused to grow, it increases with the mass of the leaf, no matter whether the shoot is situated at the base or the apex. The leaf has a powerful inhibitory effect upon the development of basal shoots. If we assume this inhibitory influence to be due to inhibitory substances we must further assume that not more than mere traces of these inhibitory substances reach the apex which are not sufficient to interfere with the growth of shoots. At the moment we cut out the piece of stem from a plant the stem contains throughout a sufficient quantity of these inhibitory substances to prevent shoots from growing, and these inhibitory substances will continue to flow in the descending sap towards the base of the stem. The most apical buds in the stem will hence be the first ones to become sufficiently free from inhibitory substances to be able to grow and the regeneration of shoots will start at the apex of the piece of stem. As soon as the shoots are beginning to grow at the apex they in turn act like a leaf so that now the further growth of shoots at the base is permanently inhibited. On the other hand, the influences which inhibit shoot formation at the base are associated or identical with influences favoring root production. Hence the leaf will favor root formation at the base of the stem and shoot formation at the apex. This gives an idea how the leaf may contribute by its "internal secretion" to the establishment of the polar character of regeneration.

If it could be shown that plants possess a closed circulatory system comparable to that of animals, all these facts might become easily intelligible if we assume that inhibitory substances for shoot formation (and favorable substances for root formation) are carried in the descending sap from the leaf to the root, where they are retained or altered, so that the ascending sap becomes practically (but not absolutely) free from these substances and contains only the nutritive material for the formation of shoots.

The assumption that the inhibitory influence of a leaf upon shoot formation in the basal part of a stem is due to inhibitory substances, is not without analogy in biology. It is known that when twins in cattle have different sex the female is in the majority of cases sterile, and Lillie[6] has shown that there exists an exchange of blood between

[6] Lillie, F. R., *J. Exp. Zool.*, 1917, xxiii, 371.

such embryos. This indicates that there exists in the blood of the male cattle embryo an inhibitory substance which prevents the normal development of the sex glands of the female embryo.

A second case is that of the prevention of the development of the male plumage in the female fowl. Boring and Pearl[7] have shown that the ovary of such females contains specific cells, the lutear cells, which are absent in the male. Boring and Morgan[8] have found that in the Sebright, where the male shows hen-feathering, lutear cells exist in the testes of the male bird. Since extirpation of the ovary in fowl and duck leads to the assumption of the full male plumage by the female (Goodale[9]), it seems as if some specific substance in the ovary inhibited the development of male plumage in the female. This inhibitory substance may be contained in the lutear cells, which, however, cannot well influence the development of feathers in any other way than by the secretion of some substance into the blood. The assumption that the inhibition of shoot formation in the basal part of a stem by a leaf is due to an inhibitory substance secreted by the leaf is therefore not without a precedent.

It is, however, necessary to call attention to the fact that even if the inhibitory influence of the leaf upon shoot formation should turn out to be based on the chemical character of the sap sent out by the leaf, it does not follow that all phenomena of inhibition and correlation in regeneration will find their explanation on the same basis. Quantitative experiments published in a former paper suggest that the inhibitory influence of a piece of stem on shoot formation in the leaf of *Bryophyllum calycinum* is due to the fact that the leaf sends its sap normally to the stem, and that as long as this happens the buds in the notches of the leaf cannot grow out.[10]

We shall show in the next communication that growing buds have inhibitory influences upon the formation of shoots comparable to the same influences caused by a leaf.

[7] Boring, A. M., and Pearl, R., *Anat. Rec.*, 1917, xiii, 253. Pearl and Boring, *Am. J. Anat.*, 1918, xxiii, 1.

[8] Boring, A. M., and Morgan, T. H., *J. Gen. Physiol.*, 1918, i, 127.

[9] Goodale, H. D., *Biol. Bull.*, 1910–11, xx, 35; *Am. Nat.*, 1913, xlvii, 159; *J. Exp. Zool.*, 1916, xx, 421.

[10] Loeb, *Ann. Inst. Pasteur*, 1918, xxxii, 1.

SUMMARY.

1. In *Bryophyllum calycinum* two apical leaves suppress the shoot formation in all the dormant buds situated basally from the leaf; one apical leaf suppresses the shoot formation in the basal buds situated in the same half of the stem where the leaf is, and, if one-half of the petiole of such a leaf is removed, the growth of basal buds in one quadrant of the stem is suppressed.

2. This inhibitory influence of a leaf upon shoot formation in the basal part of a stem is diminished or disappears when the mass of the leaf is reduced below a certain limit.

3. The inhibitory influence of an apical leaf upon the growth of shoots in horizontally suspended stems is greater when the leaf is on the upper than when it is on the lower side of the stem.

4. All these facts suggest the possibility that the inhibitory influence of the leaf upon shoot formation is due to inhibitory substances secreted by the leaf and carried by the sap from the leaf towards the base of the stem.

5. An apical leaf accelerates root formation in the basal part of a stem and this accelerating effect increases with the mass of the leaf.

6. This inhibitory influence of a leaf upon shoot formation and the favoring influence upon root formation in the more basally situated parts of the stem is one of the factors determining the polar character of regeneration.

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AMPHOTERIC COLLOIDS.

III. CHEMICAL BASIS OF THE INFLUENCE OF ACID UPON THE PHYSICAL PROPERTIES OF GELATIN.

BY JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, November 27, 1918.)

I.

Many of the authors who have worked on the physical chemistry of proteins, like Hardy, Pauli, Michaelis, Robertson,[1] and others, have pointed out that the different properties of proteins, *e.g.* swelling, viscosity, are affected by electrolytes in a parallel way, a fact which suggests that these variations are due to the same variable. The nature of this variable is not known and the majority of authors believe it to be connected with the colloidal character of the proteins, while others are inclined to assume a purely chemical or stoichiometrical relation. The reason for this doubt lies in the fact stated appropriately by Pauli[2] in discussing the influence of acid and alkali upon the osmotic pressure of gelatin.

Pauli and Handowski have pointed out that in these experiments too the essential feature is the formation of ionic protein. But a satisfactory explanation of this increase is still lacking, because we have no measurements of the molecular concentrations with the aid of other methods, which prove that we are dealing with a true osmotic pressure in the sense of van't Hoff.

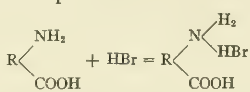
Pauli assumes that the ionized protein undergoes a stronger "hydratation" than non-ionized protein and that this hydratation explains the swelling of gelatin, as well as the apparent osmotic pressure, the latter being only a phenomenon similar to swelling.

[1] The reader is referred for the literature on the subject to Robertson, T. B., The physical chemistry of the proteins, New York, 1918.

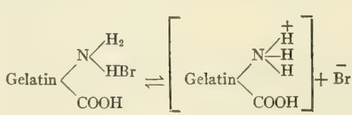
[2] Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv, 245.

It seemed to the writer that experiments on gelatin might permit us to satisfy the demand of Pauli; namely, to supply the molecular measurements necessary to show that the osmotic pressure and the other properties of gelatin solutions vary in proportion to the amount of acid combining with a given amount of gelatin. In two previous papers the writer has already shown that this is true for the influence of neutral salts on these properties of gelatin.[3] Procter's[4] experiments also indicate a purely stoichiometric basis for the influence of acids on the swelling of gelatin.

According to Werner[5] amphoteric electrolytes are characterized by their ability to add H ions or OH ions and not by their ability to give off H and OH ions. It is generally assumed, and probably correctly, that when an acid like HBr combines with an amino-acid or a protein, the reaction occurs in an NH_2 group of the amino-acid or protein. According to Werner when NH_3 and HBr combine, the positively charged hydrogen ion of HBr is attached by a secondary valency to the N whose three negative charges now hold four positively charged H ions. No other positive ion except H can act in this way. The Br is able to dissociate as freely in the NH_4Br as in the free acid. The same assumption is to be made for the way an acid, *e.g.* HBr, combines with amino-acids or proteins.



Such a molecule, *e.g.* gelatin bromide, dissociates into a positively charged gelatin ion and a negatively charged Br ion, the H ion of the HBr now forming a part of the complex and positively charged gelatin ion.



[3] Loeb, J., *J. Gen. Physiol.*, 1918, i, 39, 237.

[4] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307. Procter, H. R., and Burton, D., *J. Soc. Chem. Ind.*, 1916, xxxv.

[5] Werner, A., Neuere Anschauungen auf dem Gebiete der anorganischen Chemie, Braunschweig, 2nd edition, 1909.

Such a gelatin salt can only exchange the Br with the anion of a neutral salt and it is impossible for a complete molecule of a neutral salt like KCl to combine with the NH_2 group as has been assumed. The writer's experiments on the action of neutral salts on gelatin treated previously with acid are in harmony with the ideas of Werner and opposed to the assumption of a pentavalent N atom in the protein molecule capable of adding a whole molecule of a neutral salt. We do not know yet whether only one or more NH_2 groups in the gelatin molecule are able to bind a molecule of HBr.

Gelatin is an amphoteric electrolyte which at the isoelectric point (which for gelatin lies at pH = 4.7) is practically insoluble. When we prepare a gelatin solution and give it a hydrogen ion concentration of 2.10^{-5} (*i.e.* pH = 4.7), the solution in less than 24 hours becomes opaque on cooling in as low a concentration as 0.25 per cent and probably at any concentration; except that the opacity due to the insolubility becomes too slight in very low concentrations to be noticeable. This explains why gelatin at the isoelectric point has practically no osmotic pressure, no swelling, a minimal conductivity, viscosity, etc.

When we treat isoelectric gelatin with a limited quantity of HBr of a low concentration a certain amount of gelatin is transformed into gelatin bromide, which is soluble and dissociates electrolytically. The higher the concentration of acid used the more gelatin bromide is formed and the more molecules of gelatin go into solution, until at a certain point all the insoluble gelatin molecules are converted into soluble gelatin bromide molecules. Since a 1 per cent gelatin bromide solution should possess the same degree of electrolytic dissociation as the HBr combined with it and since a 1 per cent gelatin bromide solution on account of the high molecular weight of gelatin must be considered as a very dilute solution, we shall commit no great error in assuming a complete electrolytic dissociation of the gelatin bromide. If it is true that the increase in osmotic pressure of gelatin under the influence of HBr is merely due to an increase in the number of soluble gelatin molecules, it must be possible to show that the osmotic pressure in this case increases approximately with the number of gelatin bromide molecules formed. This we intend to prove in the present paper.

While Pauli is right in stating that gelatin treated by acid is more highly ionized than common gelatin, I cannot agree with him that it is this ionization which causes the difference in the osmotic pressure of the gelatin at the isoelectric point and gelatin treated with acid. The increase in osmotic pressure is as our numerical results will show merely the expression of the increase in the number of particles in solution and there is no need or room for the assumption that the hydratation or any other quality except the number of particles in solution has anything to do with this increase in pressure.

Since viscosity and swelling vary practically parallel with the osmotic pressure, these phenomena must also be a function of the number of particles or ions in solution.

Hardy[6] has pointed out that the ionization of a protein increases its viscosity, and the increase of viscosity of gelatin with its ionization might then account for the parallelism between the curves for the bromine number and for the viscosity of the gelatin solution.

As far as a theory of swelling is concerned, the only one possessing any quantitative basis at present is Procter's.[4]

If we can, therefore, prove that under the influence of HBr the osmotic pressure of gelatin changes in proportion with the gelatin bromide formed, we have no further reason to question the purely chemical or stoichiometrical basis of the influence of acid upon all the physical properties of gelatin.

II. Measurements without Washing.

1 gm. of finely powdered gelatin is put for 30 minutes at 15°C. into each of a series of beakers containing 100 cc. of HBr of a different concentration, varying from M/8 to M/8192. As a control 1 gm. of gelatin is put for 30 minutes at 15°C. into 100 cc. of distilled water. The powdered gelatin is then poured into a cylindrical funnel and the acid allowed to drain off. The diameter of all the funnels being the same, the height of the gelatin gives a measure for the relative amount of swelling. Each gram of gelatin is then put into 100 cc. of HBr of the same concentration with which it had been treated before and is liquefied by heating to 50°C. Immediately after melting the time

[6] Hardy, W. B., *J. Physiol.*, 1905–06, xxxiii, 251.

of outflow through an Ostwald viscometer is ascertained at a temperature of 24°C. By keeping temperature and time required for melting and time between completing this process and the viscosity measurement constant in each case comparable results are obtained.[7] The time of outflow of distilled water through the viscometer was 55 seconds. The two curves of Fig. 1 give the values for swelling and viscosity, with the logarithms of the concentration of acid used as abscissæ. Under each acid is found the pH for the gelatin solution ascertained after the viscosity determination.

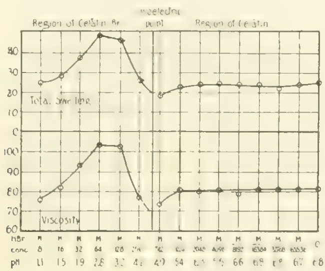


FIG. 1. Curves for viscosity and swelling of gelatin in the presence of various concentrations of HBr. Abscissæ, logarithms of concentration of HBr used. Under each concentration is found the pH of the solution. Isoelectric point and minimal values for curves at pH about 4.7. Both curves parallel, showing a maximum at pH between 2.8 and 3.2, and a drop when pH < 2.8.

The rest of the liquefied solution of gelatin in acid was then put into bags of collodion,[8] to ascertain the osmotic pressure of the gelatin solution, the pressure being expressed in mm. of height of the column of 1 per cent gelatin solution in the glass tube inserted through the tightly fitting rubber stopper closing the bag of collodion. The

[7] Loeb, *J. Biol. Chem.*, 1918, xxxiv, 395.

[8] Lillie, R. S., *Am. J. Physiol.*, 1907–08, xx, 127. Loeb, *J. Biol. Chem.*, 1918, xxxv, 497. It is hardly necessary to state that these bags are freely permeable for HBr and that HBr produces no osmotic pressure when put in such bags. There is a slight rise of the column of liquid in the manometer at the beginning of the experiment which disappears completely in a few hours, while the rise due to the gelatin, for which the membrane is impermeable, is permanent.

outside liquid was in each case HBr of the same concentration as that in which the gelatin was dissolved. The whole experiment was carried on in a water bath of a temperature of 24°C. The curve at the top in Fig. 2 is the curve for the osmotic pressure observed after about 20 hours, at which time equilibrium was established.

The conductivity of the gelatin solution was then ascertained, as well as the amount of Br found in 25 cc. of 1 per cent gelatin solution. The curves are given in Fig. 2. A glance at Fig. 2 will show that the curve for osmotic pressure is *not* parallel to the curves for conductivity and for the Br number. It is, therefore, impossible to arrive from experiments of this type at a decision whether the influence of HBr (or other electrolytes) upon gelatin is of a stoichiometrical or of a colloidal character. Yet those familiar with the literature of this subject will remember that the conclusions of most colloid chemists are based on experiments in which the action of the electrolyte upon the protein was measured in the presence of an excess of electrolyte. The second fact which deserves attention becomes clear by a comparison of Figs. 1 and 2; namely, that the minima which appear in the two sets of curves lie at different acid concentrations: in Fig. 1 between M/256 and M/512, in Fig. 2 (for osmotic pressure) between M/2048 and M/4096. Comparing, however, the pH in the two sets of curves we notice that the minimum is at the same pH, namely about 4.7, which is the isoelectric point for gelatin. A good deal of the work on which the colloidal theory of the behavior of proteins rests was done without any measurements of pH and by plotting the effect against the concentration of the acid. It is no wonder that work with two such shortcomings in its method did not furnish any proof for the stoichiometrical character of the action of electrolytes on the physical properties of amphoteric colloids.

III. Effect of Washing.

Our method consists in removing the excess of HBr (or of any other electrolyte) after it has had a chance to act on the gelatin. 1 gm. of powdered gelatin is put again into each of a series of beakers for 30 minutes at about 15° or 20°C., each beaker containing 100 cc. of HBr of a different concentration. Then the gelatin is poured on a

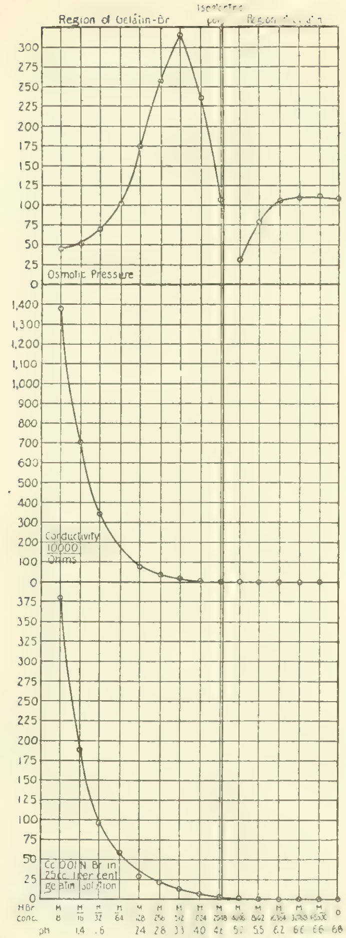
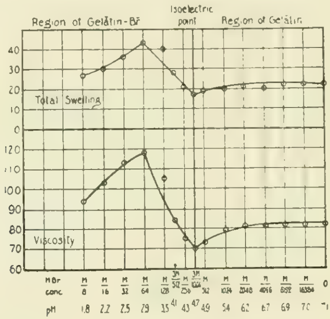


FIG. 2. Curves for bromine number (cc. 0.01 N Br in 25 cc. of 1 per cent gelatin solution), conductivity, and osmotic pressure of gelatin in presence of various concentrations of HBr. Abscissæ as in Fig. 1. No parallelism between curve for osmotic pressure and curves for bromine number and conductivity. Curve for osmotic pressure has minimum at pH about 4.6, maximum at pH = 3.3, and then drops. No conclusion can be drawn from such experiments as to the "colloidal" or true character of osmotic pressure. Osmotic pressure measured in height of column of 1 per cent gelatin solution.

filter and the acid allowed to drain off. The swelling is measured as described. From now on the method of procedure is different from that in the previously mentioned experiment. Instead of melting the gelatin in 100 cc. of the acid solution with which it had been treated, we melt it in 100 cc. of distilled water. The rest of the determinations—viscosity, osmotic pressure, conductivity, and titration for Br—are all made with such gelatin. Moreover, the osmotic pres-



FIGS. 3 and 4. Curves for viscosity and swelling (Fig. 3); bromine number, conductivity, and osmotic pressure (Fig. 4) of 1 per cent gelatin solution treated previously with different concentrations of HBr (abscissæ) the acid having been allowed to drain off. A 1 per cent solution of the gelatin *in distilled water* is then prepared, and the osmotic pressure of this gelatin is measured against distilled water, and conductivity and Br number are determined after 20 hours dialysis against distilled water. The curves for osmotic pressure, swelling, viscosity, and conductivity are parallel to the curve for bromine number from pH = 4.7 to pH = 2.9 or 3.3 respectively. The gelatin is free from bromine for pH $\gtreqless$ 4.7.

sure was measured against H_2O, thereby allowing more of the free acid not combined with the gelatin which had not drained off to diffuse out during the process. The result of this experiment is represented in Figs. 3 and 4. Fig. 4 contains the measurements for osmotic pressure and Br number, and the curves are almost parallel (with the exception of the value for the osmotic pressure for gelatin treated with M/8 acid). This parallelism is the missing link which allows us to decide in favor of the purely chemical and against the colloidal conception

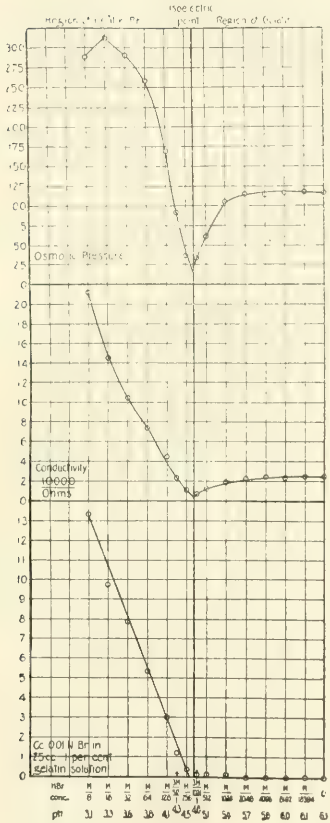


FIG. 4. See explanation under Fig. 3.

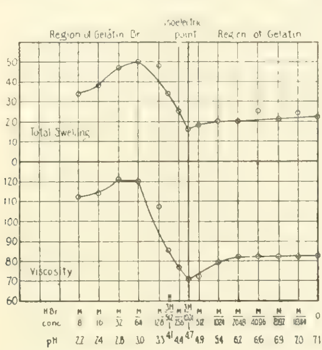
of the influence of electrolytes upon the osmotic pressure. Since the bromine number of gelatin increases parallel with the osmotic pressure (and also the viscosity and the swelling) of gelatin, these properties must depend upon the same variable; namely, the number of gelatin bromide molecules formed.

We are giving the values for the bromine number and for conductivities as actually found by analysis and measurements of resistance. These values demand, however, a correction owing to the fact that in all cases a certain amount of free HBr was present. The actually measured quantity for the bromine number is in each case the sum of the Br contained in the form of gelatin bromide and of the Br contained in the form of free HBr. The latter can be calculated from the pH. This value for the quantity of free HBr should be deducted from the Br numbers given in the curves. Since the correction thus required would be slight within the limits of pH from 4.7 to 3.6, we have omitted it in this paper. We may state, however, that the parallelism between the corrected curves for the Br number and those for osmotic pressure is even more perfect than that between our uncorrected curves for bromine number and the curves for osmotic pressure. The correction necessary for the conductivity curves can be found by measuring the resistance of a HBr solution for each pH in the same measuring cell (with fixed electrodes) which served for the measurements of the resistance of the gelatin bromine solutions. These corrections are greater than those required for the bromine number, especially for pH < 3.9. In order to obtain reliable values for conductivity we must use purified gelatin. Experiments of this kind will be reported in a subsequent paper. The conductivity measurements will not be considered in this paper.

All three curves for osmotic pressure, viscosity, and bromine number show a drop again after having reached a maximum. This drop exists in a still more pronounced way in the curves for viscosity and swelling than in that for osmotic pressure, because the gelatin contained more HBr before than after dialysis. This drop is of great theoretical significance because it shows free HBr is present in excess of the binding capacity of gelatin for HBr. The free HBr represses the ionization of gelatin bromide on account of the common Br ion and

this causes the drop in the curves for the osmotic pressure of the gelatin, since the free HBr, being able to diffuse through the collodion membrane, cannot cause any increase in osmotic pressure. The drop begins usually when pH becomes <3.3 and the drop is the more considerable the more pH falls below this level.

The correctness of this view is proved by the fact that if we wash away the traces of free acid left in the capillary spaces between the particles of gelatin after the process of draining, by perfusing the



FIGS. 5 and 6. Same curves as in Figs. 3 and 4 except that the gelatin after the acid had been allowed to drain off was washed once with 25 cc. of H_2O Parallelism between curves for Br number, conductivity, osmotic pressure (Fig. 6), viscosity, and swelling from pH = 4.7 to pH = 3.0. No Br found for pH $\gtreqless$ 4.7.

gelatin on the filter with 25 cc. of H_2O, and if we allow the water to drain off also before we make up the gelatin into a 1 per cent solution in distilled water, the drop will disappear, as is obvious from Figs. 5 and 6. In Fig. 6 the drop has disappeared, the pH going only to 3.3, and in these curves there is as complete a parallelism between the bromine number and the osmotic pressure of the gelatin solution as the strict validity of the theory of van't Hoff demands. We still observe the drop for the curves for viscosity and swelling, but the pH in these cases falls below 3.3; namely, to 2.2 (Fig. 5).

If instead of giving one washing we give four washings with 25 cc. of H_2O after the acid has drained off, we avoid the low values of pH completely and the drop in the curves for swelling disappears (Fig.

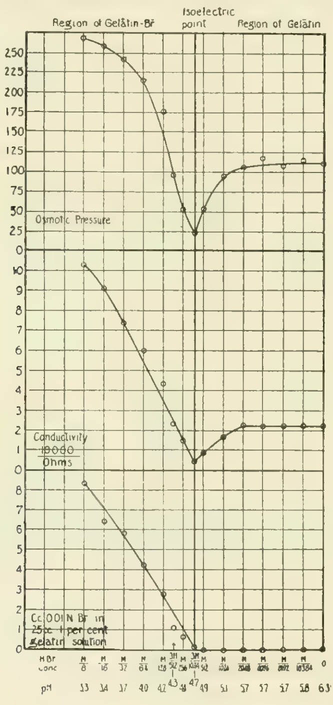


FIG. 6. See explanation under Fig. 5.

7). The parallelism between the curves for bromine number, for osmotic pressure and swelling is now practically complete.

The curves show distinctly that the independent variable is the bromine number. Thus in Fig. 7 this number was slightly in ex-

cess in the gelatin treated with M/128 and M/64 HBr. Corresponding abnormal values are found in the curves for conductivity, osmotic pressure, and swelling. The same is shown in Fig. 8. It

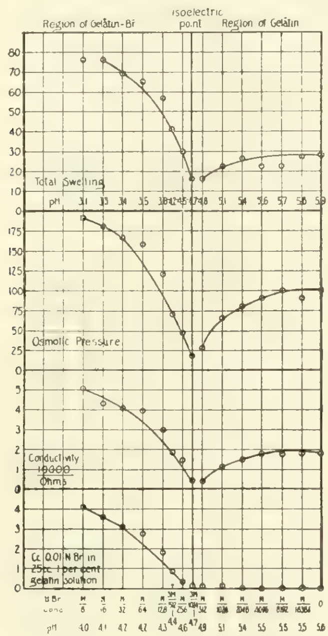


FIG. 7. Same curves as in preceding figures except that gelatin, after the acid treatment, had been washed four times with H_2O. Explanation as in preceding figures. Notice that curves are parallel and the gelatin is free from Br for pH $\geqq$ 4.7.

is, therefore, the Br number which determines the curves, *i.e.* the amount of gelatin bromide formed. Fig. 8 gives the curves for eight washings. Again the parallelism between the curves for the Br number and the other physical properties is obvious.

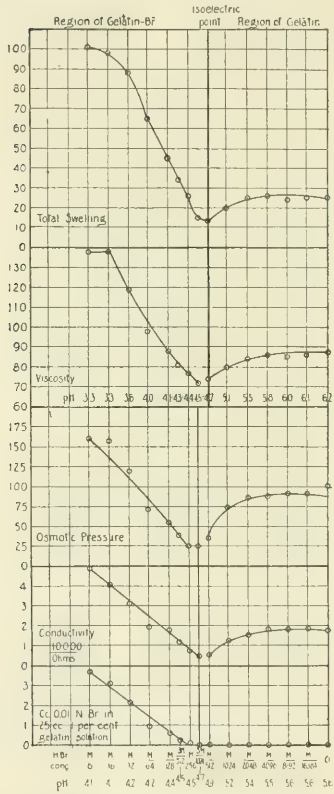


FIG. 8. Same curves as in preceding figures except that gelatin had been washed eight times with H_2O. Curves parallel and gelatin free from Br for **pH $\gtreqqless$ 4.7.**

These experiments furnish the proof that the effect of acid (HBr) upon the physical properties of gelatin is the unequivocal function of the amount of gelatin bromide formed; the probable reason for this being that pure gelatin (as it exists at the isoelectric point) is practically insoluble (and undissociated), while gelatin bromide is soluble.

We must now furnish the proof that not only is there a parallelism between the curve for the bromine number on the one hand and the curves for the physical properties of gelatin treated with HBr, but

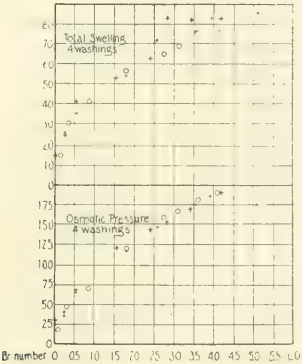


FIG. 9. The abscissæ represent the bromine number, the ordinates the values for osmotic pressure found in three different experiments with 1 per cent gelatin solution previously treated with HBr and washed four times with water. Notice agreement between values.

that to each definite Br number belongs a definite and absolute value for osmotic pressure, conductivity, swelling, and viscosity. We can do this by plotting the results of different experiments with the values for Br numbers as abscissæ and the values for osmotic pressure, swelling, etc., as ordinates. In this case the values obtained for osmotic pressure in the different experiments should differ only within the limits of the accuracy of our measurements.

In Fig. 9 the curves for three different experiments with four washings each are plotted for osmotic pressure and for swelling. The

variations lie within the limit of error. In the experiments plotted in Fig. 10 the number of washings of gelatin varied. In spite of the difference in the treatment we notice that for the same Br number practically the same value of osmotic pressure was found in all experiments. Since the curves for the other properties are parallel to the curve for osmotic pressure, it is not necessary to reproduce all the curves.

We therefore reach the conclusion that the variation of the physical properties of gelatin under the influence of HBr is an unequivocal function of the number of gelatin bromide molecules formed and that colloidal speculations not based on the laws of classical chemistry are neither needed nor warranted.

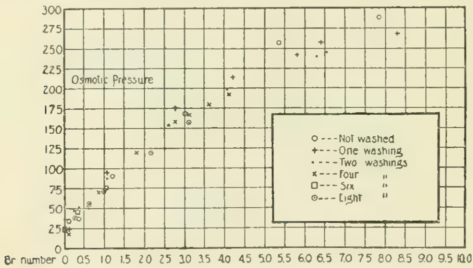


FIG. 10. Abscissæ represent bromine number, ordinates, values for osmotic pressure found in different experiments with 1 per cent gelatin solution previously treated with HBr and washed a different number of times.

IV. Titration of Gelatin with NaOH.

The following facts constitute an important link in the proof for a chemical theory of the action of electrolytes (in our case HBr) upon the physical properties of gelatin.

We notice that in all the curves the gelatin was found to be absolutely free from Br at the isoelectric point as well as on the alkaline side from the isoelectric point; *i.e.*, it was free from Br whenever pH $\geqq$ 4.7. Yet this gelatin had been treated with HBr. The fact that HBr cannot combine or remain in combination with gelatin for

pH $\geqq$ 4.7 is a further proof of the correctness of the purely chemical theory according to which for pH > 4.7 ionized gelatin can only exist as an anion, not capable of holding HBr in combination.

When we titrate 25 cc. of 1 per cent gelatin solution at the isoelectric point with 0.01 N NaOH, we find that it acts as an acid, requiring between 5.25 and 5.5 cc. 0.01 N NaOH for neutralization against phenolphthalein. (The number of cc. 0.01 N NaOH required to neutralize 25 cc. of 1 per cent gelatin we will call the NaOH number of gelatin.) When the pH of common gelatin, not treated with acid, is greater than 4.7 the NaOH number becomes less than 5.25 cc., probably on account of the fact that part of the gelatin exists as a metal gelatinate (probably chiefly Ca gelatinate) owing to ionogenic impurities remaining from the process of manufacture. Our previous papers have shown that at the isoelectric point gelatin is compelled to give off these ionogenic impurities.

On the more acid side from the isoelectric point the gelatin contains Br and the Br number increases with decreasing pH. With the exception of a small fraction this Br is held in combination with the gelatin as can be shown on the basis of titration with NaOH of the gelatin treated previously with HBr and possessing a pH < 4.7. In such a titration the gelatin solution whose pH < 4.7 is gradually rendered more alkaline through the addition of NaOH until finally its pH becomes equal to 4.7, and when that happens all the HBr held in combination with gelatin must be set free. As a consequence in a titration of gelatin bromide with NaOH two acids must be saturated with NaOH, the pure gelatin, and the HBr set free when during the process of titration the gelatin reaches its isoelectric point. It follows from this that the NaOH number found in this case must equal the sum of the Br number of the gelatin plus the NaOH number for gelatin at the isoelectric point; regardless of how the gelatin had been treated before and regardless of the pH for which this rule is tried out.

If we denote a given pH with n, the NaOH number at this pH = n as "$(NaOH)_n$," the NaOH number at the isoelectric point with "NaOH (isoelectric)," and the Br number at pH = n with "Br_n", then the following equation will hold: $(NaOH)_n$ = NaOH (isoelectric) + Br_n. In Table I, I have selected at random four experiments in which the

NaOH number and the Br number for different values of pH are given. It is easy to show that the equation holds within the limits of accuracy of our experiments. Thus the NaOH number for the isoelectric point is practically a constant in all experiments, namely between 5.25 and 5.5; and this value represents the binding capacity of "pure" gelatin for NaOH; or in other words, the binding capacity of 0.25 gm. of gelatin freed from ionogenic impurities is between 5.25 and 5.5 cc. 0.01 N NaOH, with phenolphthalein as indicator. Thus

TABLE I.

Experiment I. No washings, but made up in H_2O and dialyzed against H_2O.

pH..........	3.1	3.3	3.6	3.8	4.1	4.3	4.5	*4.8*	5.1	5.4	5.7	5.8	6.0	6.1
Br number.	13.35	9.75	7.85	5.35	3.0	1.2	0.35	*0.1*	0.1	0.1	0	0	0	0
NaOH "	18.00	15.00	13.50	11.50	9.0	8.0	7.00	*5.0*	4.0	2.5	1.75	1.5	1.5	1.5

Experiment II. One washing.

pH..........	3.3	3.4	3.7	4.0	4.2	4.3	4.4	*4.7*	4.9	5.1	5.7	5.7	5.8	6.3
Br number.	8.3	6.4	5.8	4.2	2.75	1.05	0.6	*0.1*	0	0	0	0	0	0
NaOH "	12.5	12.0	11.0	10.0	9.50	7.5	7.0	*5.5*	4.5	3.0	2.0	1.5	1.5	1.5

Experiment III. Four washings.

pH..............	3.8	4.0	4.1	4.2	4.3	4.5	*4.8*	5.0	5.3	5.6	5.7	5.8	5.9
Br number.....	4.15	3.45	2.85	2.4	0.55	0.25	*0*	0	0	0	0	0	0
NaOH "	9.00	8.50	8.00	8.0	7.0	6.00	*5.5*	4.0	2.5	2.25	1.5	1.5	1.5

Experiment IV. Eight washings.

pH..............	4.1	4.1	4.2	4.2	4.4	4.5	4.5	*4.7*	4.9	5.2	5.4	5.5	5.6
Br number.....	3.7	3.1	2.15	0.95	0.6	0.25	0.1	*0*	0	0	0	0	0
NaOH "	8.0	8.0	7.0	6.5	6.5	6.00	5.5	*5.25*	4.5	3.25	2.5	2.5	2.0

in Experiment III, for pH = 4.1, $(NaOH)_n = 8.0$, NaOH (isoelectric) = 5.5, and $Br_n = 2.85$. $5.5 + 2.85 = 8.35$, while $(NaOH)_n$ actually found is 8.0. Table II gives a comparison of the agreement in all the experiments.

In Table II we call the sum of NaOH (isoelectric) + Br_n the calculated and $(NaOH)_n$ the observed value for a given pH. The table shows that the calculated and observed values agree within the limits of the degree of accuracy of the experiments.

We have now to make sure that the HBr measured in these titrations

is practically the HBr which was in combination with the gelatin, and not free HBr left in the capillary spaces between the particles of powdered gelatin after the treatment of the latter with HBr. The reader will remember that in Experiment I (Table I) the acid was allowed to drain off and that the gelatin solution was afterwards dialyzed for about 20 hours against distilled water. In the other experiments, in addition to this the last traces of acid were removed by one or more additional washings with distilled water. It is a striking fact that for pH $\geqq$ 4.7 the gelatin was always free from Br although it had been treated with HBr. This coincidence of the point where Br begins to appear in the gelatin with the value pH $<$ 4.7 where it theoretically should begin to appear speaks already against the assumption that the Br number is the expression of free HBr not drained or dialyzed or washed off.

TABLE II.

Experiment I.			Experiment II (two washings).			Experiment III (four washings).			Experiment IV (eight washings).		
pH	$(NaOH)_n$		pH	$(NaOH)_n$		pH	$(NaOH)_n$		pH	$(NaOH)_n$	
	Calculated.	Found.		Calculated.	Found.		Calculated.	Found.		Calculated.	Found.
4.7	5.5		4.7	5.5		4.7	5.5		4.7	5.5	
4.5	5.85	7.0	4.4	6.1	7.0	4.5	5.75	6.0	4.5	5.35	5.5
4.3	6.7	8.0	4.2	8.25	9.5	4.2	7.9	8.0	4.5	5.5	6.0
4.1	8.5	9.0	4.0	9.7	10.0	4.1	8.3	8.0	4.4	5.85	6.5
3.8	10.8	11.5	3.7	11.3	11.0	4.0	9.0	8.5	4.2	6.2	6.5
3.6	13.3	13.5	3.4	11.9	12.0	3.8	9.6	9.0	4.2	7.4	7.0
3.3	15.2	15.0	3.3	13.8	12.5				4.1	8.3	8.0
3.1	18.8	18.0							4.1	8.9	8.0

The direct proof lies, however, in a comparison between the pH and the Br number. We select at random in Experiment III (Table I) pH = 4.0. The Br number found in 25 cc. of gelatin solution is for this pH 3.45 cc. 0.01 N Br. In the form of free acid this Br number would represent a hydrogen ion concentration of $\frac{3.45}{2,500}$ N, which is approximately $\frac{1}{700}$ N, while the actual normality of the gelatin solution was $\frac{N}{10,000}$; *i.e.*, less than one-tenth of $\frac{N}{700}$ (about 7 per cent). Hence more than 90 per cent of the HBr existed in chemical com-

bination with the gelatin and the small amount of free acid found was probably due to hydrolytic dissociation of gelatin bromide or to a trace of HBr not removed. This quantity of free acid is the correction of the value for the bromine number referred to in an earlier part of this paper. When, however, the free HBr is not washed off, as was the case in the experiment represented in Fig. 1, or when the free acid is only incompletely removed, the value $(NaOH)_n$ will represent, of course, more or less free acid and in this case the discrepancy between $(NaOH)_n$ and the pH found will be correspondingly smaller (see Fig. 1). This, however, manifests itself by the fact that a further rise in the Br number is no longer accompanied by a corresponding rise or is accompanied by a drop in the curves for osmotic pressure, swelling, and viscosity.

We may, therefore, consider it as proved that the bromine numbers given in this paper represent practically the HBr held in chemical combination by the gelatin with the exception of the small amount to be deducted owing to the presence of free HBr which can be calculated from the pH.

V. Theoretical Remarks.

Our experiments show that the influence of hydrobromic acid upon the physical properties of gelatin has a purely chemical or stoichiometrical basis. Gelatin and probably all proteins and amphoteric colloids behave as if they were merely amphoteric electrolytes capable of adding a H or OH ion.

Whether a protein adds an acid or a base depends on the hydrogen ion concentration; when the hydrogen ion concentration exceeds a critical point (which for gelatin is $C_H = 2.10^{-5}$), the gelatin will add acid; when it is lower it will add base. This critical hydrogen ion concentration is the isoelectric point. When gelatin is at the isoelectric point, it is free from ionogenic impurities and this "pure" gelatin is practically insoluble and hence can have no osmotic pressure, and, moreover, all the properties which depend upon its solubility are a minimum. When it is transformed into a salt by the addition of an acid (or a base) it becomes soluble, provided it is in combination with a monovalent ion, like Br or Na, etc. When pure gelatin is

transformed into a salt with monovalent anion or cation, all those properties which depend upon the number of gelatin molecules in solution increase with the proportion of gelatin salt formed, the maximum being reached when all the insoluble gelatin is transformed into soluble gelatin salt. This explains the parallelism between the curves for the bromine number of gelatin treated with HBr and the curves for the osmotic pressure of the solution. These molecular data must explain also the parallelism between the curves for viscosity and swelling with that of the bromine number.

The view taken in this paper that the osmotic pressure of protein solutions obeys the laws of classical physical chemistry is shared by one of the greatest authorities in this field, namely Sörensen.[9] Sörensen worked on egg albumin of a well defined composition which necessitated the investigation of the osmotic pressure of gelatin in the presence of ammonium sulfate. In spite of the great theoretical and experimental difficulties, which only a master like Sörensen could succeed in overcoming, he arrived at constant values for the osmotic pressure and the molecular weight of egg albumin. He states:

"The properties of colloidal solutions can be most efficiently inquired into by application, as far as possible, of the same views and methods as those generally applied to true solutions. . . .[10]

"Colloidal chemistry in the shape which has been given it by its energetic champion Wo. Ostwald offers, no doubt, to protein study a system organized with great talent, but exact experimental investigation has not been able to keep up with the systematic treatment, and therefore the value of the contents does not always correspond with the perfection of the system itself. Thus I disagree with Ostwald, who . . . warns us against a comparison of the circumstances in colloidal and real solutions. In the case of albumin solutions, and doubtless also several other typical emulsoid systems, such a comparison between the properties of the colloidal solution and those of a real solution is of the greatest significance for the right understanding of the character of the colloidal solution. Indeed, I think it is even possible to go one step further and to say that the study of real solutions may derive considerable advantage from the results which an exact research of well-defined protein solutions can give, the colloidal character of these permitting the use of research-methods—I refer especially to the use of

[9] Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1917, xii.

[10] Sörensen, *Compt. rend. trav. Lab. Carlsberg*, 1917, xii, 369.

semi-permeable membranes—, which, when dealing with real solutions, can only be used in exceptional cases and under especial circumstances."[11]

SUMMARY.

1. The method of removing the excess of hydrobromic acid after it has had a chance to react chemically with gelatin has permitted us to measure the amount of Br in combination with the gelatin. It is shown that the curves representing the amount of bromine bound by the gelatin are approximately parallel with the curves for the osmotic pressure, the viscosity, and swelling of the gelatin solution. This proves that the curves for osmotic pressure are an unequivocal function of the number of gelatin bromide molecules formed under the influence of the acid. The cc. of 0.01 N Br in combination with 0.25 gm. of gelatin we call the bromine number.

2. The explanation of this influence of the acid on the physical properties of gelatin is based on the fact that gelatin is an amphoteric electrolyte, which at its isoelectric point is but sparingly soluble in water, while its transformation into a salt with a univalent anion like gelatin Br makes it soluble. The curve for the bromine number thus becomes at the same time the numerical expression for the number of gelatin molecules rendered soluble, and hence the curve for osmotic pressure must of necessity be parallel to the curve for the bromine number.

3. Volumetric analysis shows that gelatin treated previously with HBr is free from Br at the isoelectric point as well as on the more alkaline side from the isoelectric point (pH $\geqq$ 4.7) of gelatin. This is in harmony with the fact that gelatin (like any other amphoteric electrolyte) can dissociate on the alkaline side of its isoelectric point only as an anion. On the more acid side from the isoelectric point gelatin is found to be in combination with Br and the Br number rises with the pH.

4. When we titrate gelatin, treated previously with HBr but possessing a pH = 4.7, with NaOH we find that 25 cc. of a 1 per cent solution of isoelectric gelatin require about 5.25 to 5.5 cc. of 0.01 N NaOH for neutralization (with phenolphthalein as an indicator).

[11] Sörensen, *Compt. rend. trav. Lab. Carlsberg*, 1917, xii, 5–6.

This value which was found invariably is therefore a constant which we designate as "NaOH (isoelectric)." When we titrate 0.25 gm. of gelatin previously treated with HBr but possessing a $pH < 4.7$ more than 5.5 cc. of 0.01 N NaOH are required for neutralization. We will designate this value of NaOH as "$(NaOH)_n$," where n represents the value of pH. If we designate the bromine number for the same pH as "Br_n" then we can show that the following equation is generally true: $(NaOH)_n = NaOH\ (\text{isoelectric}) + Br_n$. In other words, titration with NaOH of gelatin (previously treated with HBr) and being on the acid side of its isoelectric point results in the neutralization of the pure gelatin (NaOH isoelectric) with NaOH and besides in the neutralization of the HBr in combination with the gelatin. This HBr is set free as soon as through the addition of the NaOH the pH of the gelatin solution becomes equal to 4.7.

5. A comparison between the pH values and the bromine numbers found shows that over 90 per cent of the bromine or HBr found was in our experiments in combination with the gelatin.

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AMPHOTERIC COLLOIDS.

IV. THE INFLUENCE OF THE VALENCY OF CATIONS UPON THE PHYSICAL PROPERTIES OF GELATIN.

BY JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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I. INTRODUCTION.

In 1901 and 1902 the writer[1] published a series of investigations in which he showed that low concentrations of bivalent cations, practically without regard to their chemical nature, *e.g.* Mg, Ca, Sr, Ba, Zn, Co, Pb, etc., inhibited the toxic action of high concentrations of salts with univalent cations, upon the eggs of *Fundulus*. Trivalent cations like $AlCl_3$ and $CrCl_3$ seemed to act still more effectively than the bivalent ions though it was obvious that secondary influences (*e.g.* the high hydrogen ion concentration) restricted the limit of their antagonistic influence. This influence of valency the writer attributed to the effect of the electric charges of the ions upon the physical state of the colloidal material. He also found that polyvalent anions, *e.g.* SO_4, oxalate, citrate, etc., had under the same conditions no antagonistic effect. Since that time cases of an antagonistic action of polyvalent anions have come to light, but this phenomenon is not only less common but also less striking than the antagonistic effect of polyvalent cations.

It had been shown in the writer's preceding publications on gelatin[2] that the salts of gelatin with univalent cations possess a comparatively high osmotic pressure, a high viscosity, a high degree of swell-

[1] Loeb, J., *Arch. ges. Physiol.*, 1901–02, lxxxviii, 68; *Am. J. Physiol.*, 1901–02, vi, 411.

[2] Loeb, J., *J. Biol. Chem.*, 1918, xxxiii, 531; xxxiv, 77, 395, 489; xxxv, 497; *J. Gen. Physiol.*, 1918–19, i, 39, 237, 363.

ing, and a high alcohol number, while the gelatin salts with bivalent cations have a much lower osmotic pressure, lower viscosity, etc. It was also found that the addition of a certain amount of a salt with a bivalent or polyvalent cation depresses the effect of salts with monovalent cation on osmotic pressure, viscosity, swelling, etc., of the gelatin.

Pauli[3] and Michaelis[4] seem to ascribe the variations in the swelling and the viscosity of protein solutions to variations in the degree of ionization and to a "hydratation" they assume to be connected with the ionization of the protein. According to this view we should have to assume that sodium gelatinate has a higher osmotic pressure than calcium gelatinate of the same concentration, because the former is more strongly ionized. In an earlier paper the writer tentatively accepted Pauli's hypothesis, but a closer scrutiny of the literature showed that neither Pauli nor Michaelis measured the effect of electrolytes upon the conductivity of their protein solutions, probably on account of the fact that they did not remove the excess of electrolyte after it had acted on the protein. The writer's method of removing the excess of electrolytes after they have had time to react with the gelatin made measurements of conductivity possible, and these measurements in connection with measurements of osmotic pressure and of the quantity of metal in combination with the gelatin led to a very definite explanation of the influence of the valency of ions on the properties of gelatin. *With the same equivalent of metal in combination with a given mass of gelatin the maximal osmotic pressure of a 1 per cent solution of gelatin salts with univalent cation, e.g. Na gelatinate, is almost exactly three times as great as that of gelatin salts with a bivalent metal, e.g. Ca gelatinate, while the conductivities of the solutions of the two types of gelatin differ little or not at all.* This indicates that the gelatin salts with univalent metal have at the point of maximal osmotic pressure about three times as many particles in solution as the same mass of gelatin salts with bivalent metal, while the number of electrical charges is about the same in both cases. The identity of the conductivities of gelatin salts of the type of sodium gelatinate and calcium

[3] Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv, 245.

[4] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.

gelatinate proves that the difference in the influence of the valency of the cation upon the physical properties of gelatin cannot be ascribed to a difference in the degree of ionization of the two types of salts, but is due to some other as yet unknown factor. It seems possible to explain all the phenomena on the basis of the tentative assumption that gelatin salts with a bivalent cation dissociate into cations and aggregates of four, or six, or eight gelatin ions, each individual gelatin ion of the aggregate retaining its original negative charge, while the anions of salts of sodium gelatinate consist of only one gelatin ion each. The quantitative data suggest that the number of gelatin anions contained in each aggregate is of a stoichiometrical order, being a simple multiple of the number representing the valency of the polyvalent metal ion.

II. Amount of Alkali Combining with Gelatin.

We used powdered Cooper's non-bleached gelatin which is impure, having a pH of about 7.0 and consisting to a large extent of calcium gelatinate. It is necessary to purify this gelatin before using it by bringing it to the isoelectric point pH = 4.7.[5] This is done by putting 1 gm. of gelatin for $\frac{1}{2}$ hour into 100 cc. of 3 M/1024 HCl or M/128 acetic acid, then putting the gelatin on a filter, allowing the excess of solution to drain off, and washing the gelatin two or three times with 25 cc. of distilled water at 5°C. If we wish to transform the pure isoelectric gelatin into a metal gelatinate we treat it subsequently with the hydroxide of the metal—*e.g.* NaOH, $Ca(OH)_2$, etc.—with which we wish the gelatin to combine.

We always used finely powdered gelatin rendered isoelectric in the manner described. When we intended to prepare sodium gelatinate we treated different doses of isoelectric gelatin of 1 gm. each with 75 or 100 cc. of a NaOH solution varying from M/4 to M/8192 NaOH, after-

[5] Isoelectric gelatin does not react with neutral salts like NaCl but will react with NaOH, since in this latter case the pH is raised beyond that of the isoelectric point. Common Cooper's gelatin, with a pH = 7.0, consisting of gelatin salts, especially Ca gelatinate, will be influenced in the same way by a treatment with NaOH as with NaCl, since in both cases a replacement of Ca by Na will occur. This is supported by the writer's previously published papers.

wards washing away the excess of alkali. In each case sodium gelatinate was formed but the amount formed varied with the concentration of the alkali solution used; and the pH varied correspondingly. It was of importance to measure the amount of Na or Ca in combination with the gelatin, to make sure that we were dealing with phenomena of a stoichiometrical character; it was especially necessary to make sure whether or not Na and Ca combine with gelatin in equivalent proportions. The metal gelatinate used for this purpose was not only washed as indicated, but was also dialyzed over night through collodion bags against 400 cc. of distilled water.

Our former experiments[6] allow us to measure the amount of metal contained in a given mass of gelatin when the pH is known. Metal gelatinates can only exist on the alkaline side from the isoelectric point of gelatin, this point being defined by a pH = 4.7. The Na or K or Ca in combination with the gelatin at each pH can be calculated in the following way. We determine the cc. of 0.01 N NaOH required to bring 25 cc. of 1 per cent isoelectric gelatin of different pH (lying between 4.7 and 7.0) to the point of neutrality (pH = 7.0). By deducting this value from the quantity required to bring 25 cc. of 1 per cent gelatin solution from the isoelectric point to pH = 7.0—which was always found to be about 4.5 cc. of 0.01 N NaOH[7]—we obtain the amount of 0.01 N Na or of $\frac{1}{2}$ Ba or $\frac{1}{2}$ Ca in combination with the 25 cc. of 1 per cent gelatin at any pH between 4.7 and 7.0. When pH is > 7.0 we ascertain the amount of 0.01 N HCl required to bring the 25 cc. of gelatin to pH = 7.0, and add this to the value 4.5. In Fig. 1 the abscissæ are the pH, the ordinates the cc. of 0.01 N Na, K, or $\frac{1}{2}$ Ba found in combination with 25 cc. of gelatin for each pH. The results of three different experiments with the three different alkalies named are plotted (Fig. 1), showing the degree of agreement of the results. The curves are exactly the same whether gelatin has been treated with KOH, NaOH, LiOH, $Ba(OH)_2$, or $Ca(OH)_2$. It is also obvious from the curves that we are dealing with a simple salt formation of

[6] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 363.

[7] In our preceding paper we determined the cc. of 0.01 N NaOH required to bring 25 cc. of 1 per cent gelatin from the isoelectric point to the turning point of phenolphthalein (pH = 9.0) and found 5.5, which agrees with our present result where we neutralize to pH = 7.0 instead of to 9.0.

a stoichiometrical nature and that one Ba or Ca replaces two Na (or two Li, K, or NH_4). The maximum amount of salt formed is practically reached not far beyond pH = 8.0, which is so near the point of neutrality that practically no corrections for the values for titration and only slight corrections for the values for conductivity are required. Our conductivity curves are the corrected curves, *i.e.* from the measured values the conductivities of the pure alkali solutions of the same pH are deducted.

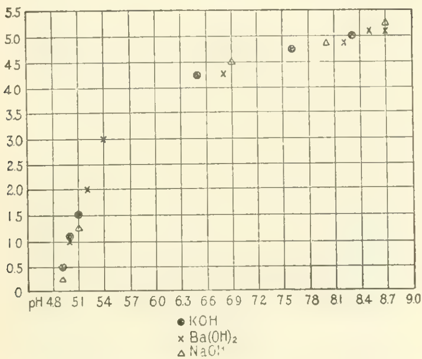
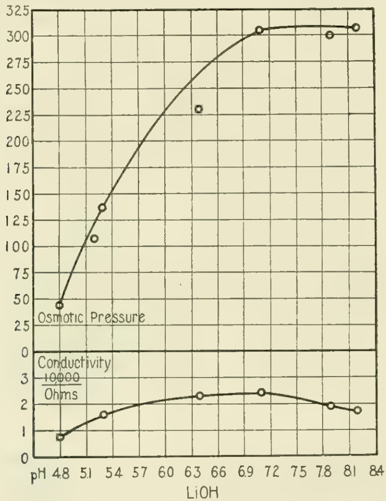


FIG. 1. Ordinates represent amount of Na, K, and ½ Ba (expressed in cc. of 0.01 N Na) in combination with 25 cc. of 1 per cent gelatin solution, previously treated with NaOH, KOH, or $Ba(OH)_2$ respectively and freed from the excess of salt by washing. Abscissæ represent pH of the solution. Curves in all three cases are identical, showing that one Ba replaces two Na or K.

III. Action of Different Alkalies on the Conductivity and Osmotic Pressure of Gelatin.

In order to obtain constant results we brought the powdered gelatin, as stated, to the isoelectric point by treating it for 30 minutes with M/128 acetic acid at 20°C. The gelatin was then put on a filter, the acid allowed to drain off, and then it was washed twice with 25 cc. of distilled water at about 5°C. Then such gelatin, while on the filter, was perfused three times with 25 cc. of a solution of NaOH or KOH,

etc., of a definite concentration, varying from M/4 to M/8192 at 15°C. During this perfusion the powdered gelatin was sufficiently stirred to allow intimate contact between all the gelatin particles and the alkali, and then after all the excess of alkali had been allowed to drain off the mass on the filter was washed once with 25 cc. of H_2O at 5° and



FIGS. 2 to 7. Curves for osmotic pressure (in terms of mm. of a 1 per cent gelatin solution) and conductivity $\left(\frac{10{,}000}{\text{ohms}}\right)$ of a 1 per cent gelatin solution first rendered isoelectric and then treated with concentrations of an alkali, *e.g.* NaOH, varying from M/8 to M/8192 to cause a varying proportion of the gelatin to form a metal gelatinate. Abscissæ represent pH of the solution after dialysis; ordinates of upper curve, osmotic pressure, and of lower curve, conductivity of the solution found at different pH. Curves for the osmotic pressures for gelatin salts with univalent ion, Li, Na, K, and NH_4 (Figs. 2 to 5) are alike and high, reaching a maximum of about 325 mm. Curves for the osmotic pressures of salts with bivalent metals, Ca and Ba, are very much lower than those for metal gelatinates with univalent metal, reaching a maximum of only 125 mm. Curves for conductivity are almost identical for both types of gelatin salts.

once with 25 cc. of H_2O at 20°C. Then the mass was made into a 1 per cent solution and put into collodion bags to determine the osmotic pressure. Each collodion bag was surrounded by a beaker containing 400 cc. of distilled water and the temperature was kept constant at 24°C. This allowed any excess of alkali left to diffuse out of the bag. The next day the osmotic pressure was measured, the pH of the gela-

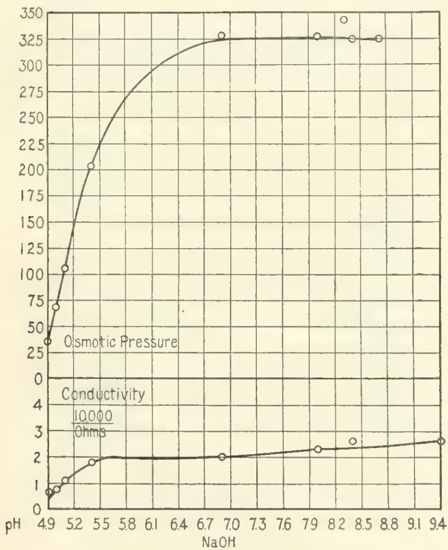


FIG. 3. See explanation under Fig. 2.

tin solution was determined, the solution titrated to determine the quantity of Na or K, etc., in combination with the gelatin, and the conductivity of the solution was measured.

The results of these measurements are contained in Figs. 2 to 7. The abscissæ are the pH, the ordinates the values for conductivity and osmotic pressure for these pH. The reader will notice that the curves representing the influence of Li, Na, K, and NH_4 on the osmotic

pressure (and the other physical properties) of gelatin are identical, if we use the pH as abscissæ. This result contradicts the statements current in colloid chemistry according to which these four cations have a different effect. The colloid chemists who make such statements have failed to measure the hydrogen ion concentration of their solutions. Our experiments show that the effects of the same cation on

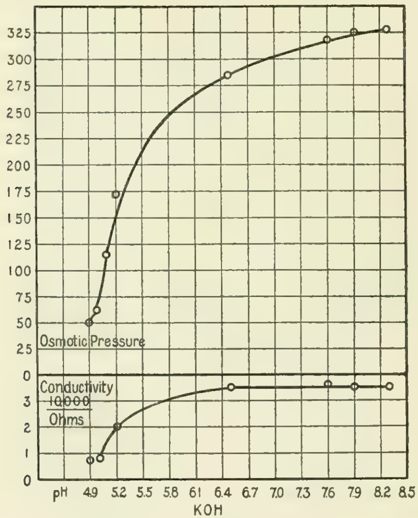


FIG. 4. See explanation under Fig. 2.

gelatin differ for different pH and hence we cannot be sure that apparent differences in the effect of two cations on a protein are the expression of differences in the structure of the two cations, unless we are certain that the pH is the same in both cases. The colloid chemists have, moreover, compared the effects of neutral salts of Li, Na, K, and NH_4, in the presence of an excess of these salts, which introduces a second error.

It is obvious that the osmotic pressure in the curves for LiOH, NaOH, KOH, and NH_4OH (Figs. 2 to 5) reaches the same maximum (at pH between 7.0 and 8.0), namely that of a column of about 325 mm. of a 1 per cent gelatin solution; and that the curves for osmotic pressure of the Ca gelatinate and Ba gelatinate (Figs. 6 and 7) reach also an equally definite maximum of about 125 mm. osmotic pressure at about the same pH. In both cases we have probably to deduct

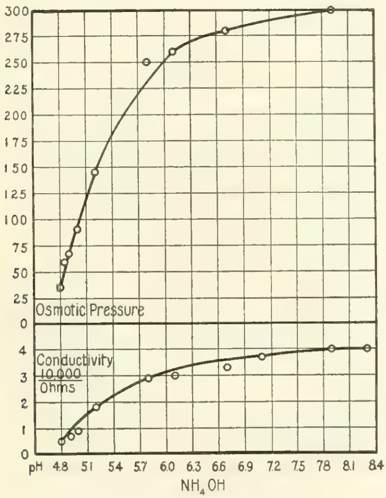


FIG. 5. See explanation under Fig. 2.

from these values about 25 mm.; namely, the osmotic pressure of a 1 per cent gelatin solution at the isoelectric point which includes the necessary capillary correction. This then leaves the following characteristic and constant values for the maximum osmotic pressure of the two types of metal gelatinates:

Li, Na, K, NH_4 gelatinate	300 mm.	(uncorrected value 325 mm.)
Ca and Ba gelatinate	100 "	(" " 125 ")

The constant character of this ratio of the two pressures of 1 : 3 (or 3 : 8 for the uncorrected value) for the two valencies, but regardless of the other qualities of the ions, betrays a stoichiometrical basis for the influence of valency. The experiments were repeated to guard against error, the results remaining the same.

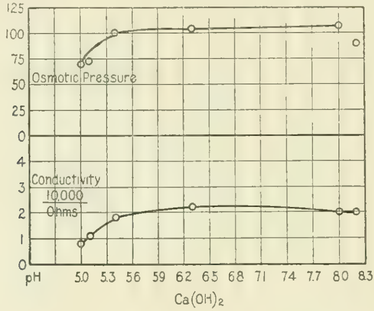


FIG. 6. See explanation under Fig. 2.

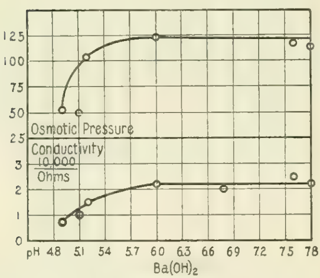


FIG. 7. See explanation under Fig. 2.

This means that in a 1 per cent solution of a metal gelatinate we have approximately three times as many particles in solution or suspension when the metal is univalent as when it is bivalent. Before we can draw any further conclusions we have to consider the relative conductivities of the same gelatin solutions.

A glance at the curves for conductivity shows that those for Na, Li, Ba, and Ca are almost identical, while the curves for K and NH_4 are a little higher than the others. These experiments were repeated and the same values were obtained. Fig. 8 shows that while the curves for conductivity of gelatin salts with univalent and bivalent metals (Na and Ba) are almost identical, the curves for the osmotic pressure of the two types of salts are very different.

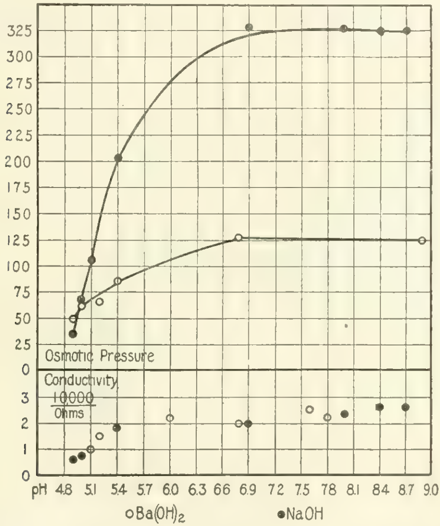


FIG. 8. Showing that while the curves for conductivity of sodium and barium gelatinate are practically identical, the curves for the osmotic pressures are very different.

There are two possible explanations for the fact that the ratio of conductivities of the two types of salts (Ca gelatinate and Na gelatinate) is 1 : 1 while the ratio of osmotic pressures is 1 : 3. The one explanation is that the degree of electrolytic dissociation of Ca gelatinate is so much smaller than that of Na gelatinate as to produce a

ratio of 1:3 particles in solution. This would account for the difference in the ratio of osmotic pressures but would leave unexplained the identity of conductivity of the two solutions. It would therefore be necessary to make a second assumption; namely, that the lower viscosity of a 1 per cent Ca gelatinate solution would raise the conductivity of a Ca gelatinate solution enough to compensate for the smaller degree of electrolytic dissociation.

In order to account for the ratio of 1:3 in osmotic pressure of the solutions of the two types of salts we have to assume that only about 20 to 25 per cent of the Ca gelatinate molecules are dissociated, while the dissociation of the sodium gelatinate is complete (five molecules of Ca gelatinate, one of which dissociates, would yield seven particles while the same amount of gelatin would form ten molecules of sodium gelatinate, yielding with complete dissociation twenty particles; this would result in a ratio of 7:20 for the relative number of particles in solution). The four electric charges of the one dissociated Ca gelatinate molecule would have to give the same conductivity as the twenty electric charges of the sodium gelatinate.

Our present knowledge speaks against such an influence of the viscosity of gelatin solutions upon conductivity. We prepared 1 per cent solutions of sodium, potassium, magnesium, and calcium gelatinate, of pH = 7.0, by putting 1 gm. of finely pulverized commercial Cooper's gelatin (probably mostly calcium gelatinate) for 1 hour at 20°C. into 100 cc. of M/4 NaCl or KCl, or $MgCl_2$ or $CaCl_2$, and allowed the excess of salt solution to drain off by putting the gelatin on a filter. We then washed the gelatin on each filter six times in succession with 25 cc. of H_2O, melted the gelatin by heating to about 50°C., and added enough water to make a 1 per cent gelatin solution. The solution was cooled to 24°C. and the time of outflow through a viscometer, as well as the conductivity of each solution, was measured immediately, at 24°C. (Table I). We found the usual typical difference in viscosity between Ca and Mg gelatinate on the one hand, and Na and K gelatinate on the other. It is well known that the viscosity of a gelatin solution prepared by melting will increase on standing, especially at a low temperature. The gelatin solutions were kept at about 2°C. for 2 hours and were then heated to 24°, and their viscosity and conductivity were again measured. All the

viscosities had increased considerably and the viscosity of magnesium and calcium gelatinate was now as great as was originally that of the sodium gelatinate. Yet the conductivities were practically unaltered. The experiment was continued as indicated in Table I and enormous viscosities resulted, practically without any increase in the conductivities.

The reader will notice incidentally from the continuation of the experiment that upon heating to 50°C. and cooling to 24° the viscosity went practically back to its original level for the four different gelatin salts. In this case the influence of the "history" upon the colloidal solution is entirely reversible.

TABLE I.

Variation of Viscosity and Conductivity of Gelatin Solutions upon Standing. Viscosity in Seconds of Outflow, Conductivity, $\frac{10{,}000}{Ohms}$. All Measurements at 24°C.

Treatment.	Na gelatinate.		K gelatinate.		Mg gelatinate.		Ca gelatinate.	
	Viscosity.	Conductivity.	Viscosity.	Conductivity.	Viscosity.	Conductivity.	Viscosity.	Conductivity.
	sec.	$\frac{10{,}000}{ohms}$	sec.	$\frac{10{,}000}{ohms}$	sec.	$\frac{10{,}000}{ohms}$	sec.	$\frac{10{,}000}{ohms}$
Immediately after melting...	99	2.31	102	3.07	88	2.10	86	2.12
After 1 hr. in refrigerator and heating to 24°C.......	135	2.22	143	2.94	138	2.06	130	2.12
After 18 hrs. in refrigerator and heating to 24°C.......	180	2.28	250	3.03	240	2.07	200	2.09
After being kept at 24° for 2 hrs....................	170	2.35	210	3.09	194	2.10	173	2.13
After heating to 50° and cooling to 24°............	94.5	2.31	95.5	3.11	86	2.12	83.5	2.14

These experiments seem to exclude the assumption that the degree of electrolytic dissociation of calcium gelatinate is so much smaller than that of sodium gelatinate that it produces a ratio of 1:3 in the osmotic pressure of the two solutions; and that the difference in the degree of dissociation is compensated by the influence of viscosity upon conductivity in such a way as to make the conductivities of the solution of the two types of salts equal.

The second possible explanation is based on the assumption that the equality of conductivity is due to the fact that both types of solutions in equal concentration and for the same pH possess an approximately equal number of charges.

The identity of equivalents combining with gelatin demands that twice as many gelatin molecules must combine with one atom of Ca as with one atom of Na. For the sake of simplification we assume that one Na atom combines with one gelatin atom. This would mean that calcium gelatinate exists in the form of Ca $gelatin_2$ or Ca_2 $gelatin_4$ or Ca_3 $gelatin_6$, and sodium gelatinate in the form of Na gelatin. In this case all phenomena will find their explanation if we assume that in the dissociation of Ca_2 $gelatin_4$ the four gelatin ions remain aggregated in one group with four negative charges

$$Ca_2 \text{ gelatin}_4 \rightleftharpoons \overset{++}{Ca} + \overset{++}{Ca} + [\text{gel}\bar{\text{a}}\text{tin}]_4$$

Such a dissociation would therefore yield three ions, one of which contains an aggregate of four negative gelatin ions. In order to obtain the same number of charges, four molecules of Na gelatin would be required, dissociating into four positive Na ions and four separate negative gelatin ions, making eight ions in all. This would demand a ratio of osmotic pressures for the two gelatin solutions of 3:8, which is slightly less than the ratio observed. The electrical charges would be the same for the two solutions and the conductivities would only show the difference due to differences in the ionic mobilities.

If the dissociating complex in the case of calcium gelatinate is Ca_3 $gelatin_6$ resulting in the formation of three Ca ions and one aggregate $gelatin_6$ anion carrying six charges, the same number of charges would be carried by six molecules of sodium gelatinate dissociating into twelve ions. This would yield exactly the ratio of 1:3 for the osmotic pressure of solutions of calcium gelatinate and sodium gelatinate of the same concentration and conductivity.

If the aggregates consist of eight gelatin anions with four Ca ions the ratio of osmotic pressures would be 5:16 which is also approximately 1:3.

It is in reality only necessary to assume the existence of compounds of the form Ca $gelatin_2$, the two anions of which form one aggregate of two gelatin anions, and to assume further that two, three,

or more such aggregates of two gelatin anions join to form larger aggregates of four, six, or eight gelatin anions, every one of which keeps its original charge. This would account for all the phenomena observed.

We are only able to estimate the relative difference in the mobilities of the cations. They are higher for K and NH_4 than for Na and Li, and we also find that the observed conductivities of K and NH_4 gelatinate in our experiments are higher than those of Na and Li gelatinate, possibly to the amount the difference in mobility of the ions named demands.

Bayliss in comparing the osmotic pressure and the electrolytic dissociation of solutions of Congo red found that it is ionized 80 per cent in a dilution of 500 liters, yet,

"The osmotic pressure [of such solutions] found experimentally, both by direct measurement and by vapour pressure, is, throughout a wide range of concentration, uniformly between 95 and 100 per cent. of what it would be if no dissociation existed. Since it should be from one and a half to three times this value, according to the concentration, it is plain that there are some abnormal conditions present."

Bayliss suggests an explanation similar to the one given above; namely, "the possibility of aggregated simple ions carrying the sum of the charges of their components."[8]

IV. Influence of Valency upon Swelling and Viscosity.

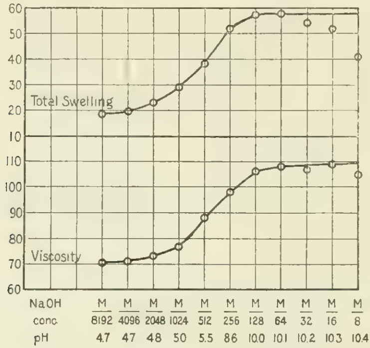
It has been demonstrated in previous papers that the curves for the values of viscosity and swelling are similar to the curves for osmotic pressure and it has been stated that these properties must also be a function of the relative number of metal gelatinate molecules and ions formed.

In Figs. 9 and 10 we give the curves for viscosity and swelling of sodium and potassium gelatinate and in Figs. 11 and 12 the same curves for barium gelatinate and calcium gelatinate. The values for viscosity are given in times of outflow and the values for swelling in terms of the height of cylinders of gelatin of the same diameter.[9] On

[8] Bayliss, W. M., *Proc. Roy. Soc. London, Series B*, 1911, lxxxiv, 253, 254.

[9] Loeb, J., *J. Biol. Chem.*, 1918, xxxiv, 77, 395.

the axis of abscissæ are the logarithms of the concentrations of the alkali solution used. The pH of the gelatin is given under each concentration. The values for Na and K gelatinate are practically iden-



FIGS. 9 to 12. Curves for viscosity and swelling of sodium gelatinate (Fig. 9), potassium gelatinate (Fig. 10), calcium gelatinate (Fig. 11), and barium gelatinate (Fig. 12). The curves for sodium gelatinate differ from the curves for Ca and Ba gelatinate in the same sense and almost the same degree as the curves for osmotic pressure.

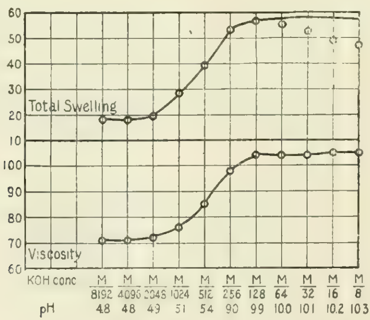


FIG. 10. See explanation under Fig. 9.

tical, and so are the values for Ca and Ba gelatinate. But the values for Na gelatinate and Ca gelatinate differ. The reader will notice that the values for pH are higher than in the curves for osmotic pressure. This is due to the fact that in the latter case the pH was determined after the excess of alkali had been removed by washing and dialysis, while the nature of the experiment made it necessary for us

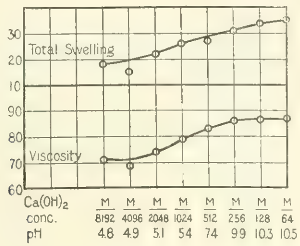


FIG. 11. See explanation under Fig. 9.

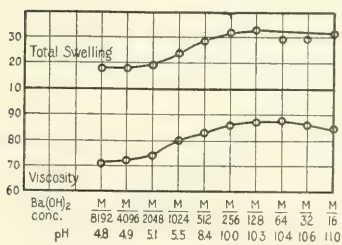


FIG. 12. See explanation under Fig. 9.

to determine the pH in the experiments on swelling and viscosity only after washing away the greater part of the excess of alkali, without using dialysis.

Neither the values for swelling nor for viscosity are as accurate as those for the osmotic pressure and hence the former cannot well serve for the purpose of drawing conclusions in regard to the molecular

basis of these properties. The viscosity rises for some time after the melting of gelatin and only approximately constant values can be obtained by measuring the time of outflow immediately; *i.e.*, within 5 minutes after the melting. The swelling is influenced by the resistance of the gelatin to the expanding forces leaving aside other sources of error. The osmotic pressure in connection with measurements on conductivity seems by far the most valuable property for the analysis of the molecular character of the influence of electrolytes upon the physical behavior of colloids.

The approximate parallelism between the curves for viscosity, swelling, and osmotic pressure excludes the idea that swelling and viscosity are determined by a hydratation due to the degree of ionization of proteins as Pauli and many other colloid chemists assume. These hypotheses were developed without measurements of conductivity.

V. Action of Trivalent Cations.

The facts of the preceding chapters furnish a suggestion for the understanding of the action of trivalent metals. If the same tentative assumption may be made, namely that the gelatin ions held by the polyvalent metal ion dissociate in one aggregate, thus causing a diminution in the number of particles present in the solution without a diminution in the number of charges, it follows that this anionic aggregate must be the greater the greater the valency of the metal ion—leaving aside the atomic volume. Hence trivalent metals like Ce or Al should, if in combination with gelatin, lead to anionic aggregates consisting of a simple multiple of three. We cannot test this inference but we can show that gelatin compounds with a trivalent metal like cerium and aluminium are practically insoluble, and this insolubility might be the consequence of the increase in the number of gelatin anions forming an aggregate with the valency of cations.

1 gm. of finely powdered Cooper's gelatin (which is essentially calcium gelatinate of pH = 7.0) was put for 1 hour into each of a series of beakers containing 100 cc. of a solution of Ce_2Cl_6 varying in concentration from M/4 to M/16,384, then drained on a filter and washed four times with distilled water to remove the excess of salt solution. The conductivity, osmotic pressure, alcohol number, and total swell-

ing were ascertained. The results are plotted in Fig. 13. Gelatin treated with Ce_2Cl_6 solution from M/4 to M/2048 becomes insoluble and the cerium gelatinate forms a precipitate. The values for conductivity, etc., were as low as those obtained for gelatin at the isoelectric point. The gelatin treated with M/4096 or less concentrated Ce_2Cl_6 was clear and gave values for conductivity, etc., rising

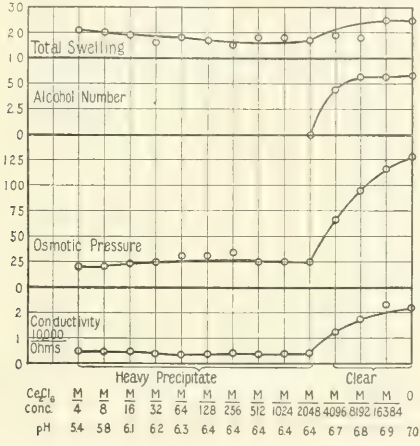


FIG. 13. Impure gelatin (chiefly calcium gelatinate) of pH 7.0 treated with concentrations of Ce_2Cl_6 varying from M/4 to M/16,384, then freed from excess of salts. pH of the gelatin solution is given under each solution. Cerium gelatinate is insoluble and hence the values for swelling, alcohol number, osmotic pressure, and conductivity are almost zero except when the concentration of the Ce_2Cl_6 used was low (M/4096 and less), when only a fraction of the original calcium gelatinate was transformed into cerium gelatinate.

with increasing solutions of Ce_2Cl_6. In these cases not all the Ca gelatinate was transformed into Ce gelatinate. The less Ce gelatinate was formed the higher the value for conductivity, osmotic pressure, alcohol number, and swelling. The pH was between 5.4 and 7.0; *i.e.*, on the alkaline side of the isoelectric point of gelatin. Lead and copper acetates gave a similar result, as was to be expected.

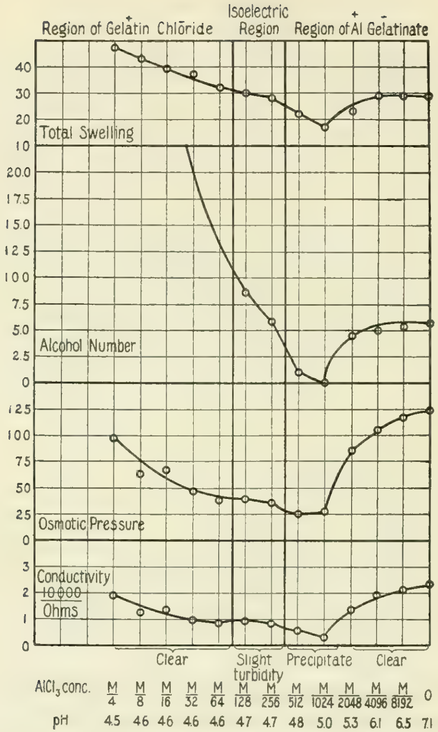


FIG. 14. Impure gelatin (chiefly calcium gelatinate) treated with different concentrations of an acid salt with trivalent cation, namely, $AlCl_3$. As long as pH < 4.7 no aluminium gelatinate is formed and the gelatin exists in the form of gelatin chloride which gives a clear solution. At the isoelectric region pH $= 4.7$ gelatin is insoluble and all the curves show the low level characteristic of gelatin at the isoelectric point. When pH > 4.7 gelatin exists as Al gelatinate. As long as the concentration of $AlCl_3$ used was high enough to transform all the original calcium gelatinate into aluminium gelatinate, M/512 to M/1024, all the gelatin was transformed into the insoluble aluminium gelatinate which formed a precipitate as in the case of cerium gelatinate. When the concentration of the $AlCl_3$ solution used was less than M/1024 not all the calcium gelatinate was transformed into aluminium gelatinate and the solution became gradually clear again.

$AlCl_3$ forms acid solutions and it seemed of interest to study the effect of a treatment of gelatin with $AlCl_3$. We know now that for pH <4.7 gelatin treated with $AlCl_3$ must form gelatin chloride and cannot be affected by the aluminium. The same is true also for pH = 4.7, where gelatin exists as sparingly soluble pure gelatin. For pH > 4.7 aluminium gelatinate can be formed which should be insoluble. If, however, the concentration of $AlCl_3$, becomes too low, less and finally practically no aluminium gelatinate should be formed. These predictions were all fulfilled as the complicated curves in Fig. 14 show. 1 gm. of Cooper's powdered gelatin (*i.e.* chiefly Ca gelatinate) was put for 1 hour at 20° into a series of beakers, each containing 100 cc. of a solution of $AlCl_3$ varying in concentration from M/4 to M/8192. The gelatin was then freed from the excess of salt solution by washing as described before. In Fig. 14 the abscissæ are the logarithms of the concentration of the $AlCl_3$ solution used and the final pH of the washed gelatin is found under each concentration. For pH <4.7 no effect of aluminium is noticeable; the curves are typical gelatin chloride curves. For pH = 4.7 we find a region where gelatin behaves like isoelectric gelatin. For pH = 4.8 and 5.0 we have a precipitate formation due to the formation of aluminium gelatinate. When the concentration of $AlCl_3$ solution used is less than M/1024 the solution becomes clear again and the curves rise with increasing dilution of the $AlCl_3$ solution used. In other words, for pH >4.7 the curves for aluminium gelatinate and cerium gelatinate run parallel as our theory demands.[10]

This curve suggests incidentally why in the writer's former experiments on *Fundulus* eggs the antagonistic influence of $AlCl_3$ to the toxic action of a sodium chloride solution was so restricted. On the acid side of the isoelectric point of amphoteric colloids $AlCl_3$ cannot act on the colloid, and on the basic side the concentration of Al is too low. It is the writer's intention to repeat these experiments with Ce_2Cl_6 or $LaCl_3$, the solutions of which are less acid.

[10] One wonders what interpretation such a curve as that for $AlCl_3$ would have found on the part of the colloid chemists who have persistently ignored not only the hydrogen ion concentration of their protein solutions but who have also overlooked the chemical significance of the isoelectric point.

SUMMARY.

1. A method is given by which the amount of equivalents of metal in combination with 1 gm. of a 1 per cent gelatin solution previously treated with an alkali can be ascertained when the excess of alkali is washed away and the pH is determined. The curves of metal equivalent in combination with 1 gm. of gelatin previously treated with different concentrations of LiOH, NaOH, KOH, NH_4OH, $Ca(OH)_2$, and $Ba(OH)_2$ were ascertained and plotted as ordinates, with the pH of the solution as abscissæ, and were found to be identical. This proves that twice as many univalent as bivalent cations combine with the same mass of gelatin, as was to be expected.

2. The osmotic pressure of 1 per cent solutions of metal gelatinates with univalent and bivalent cation was measured. The curves for the osmotic pressure of 1 per cent solution of gelatin salts of Li, Na, K, and NH_4 were found to be identical when plotted for pH as abscissæ, tending towards the same maximum of a pressure of about 325 mm. of the gelatin solution (for pH about 7.9). The corresponding curves for Ca and Ba gelatinate were also found to be identical but different from the preceding ones, tending towards a maximum pressure of about 125 mm. for pH about 7.0 or above. The ratio of maximal osmotic pressure for the two groups of gelatin salts is therefore about as 1: 3 after the necessary corrections have been made.

3. When the conductivities of these solutions are plotted as ordinates against the pH as abscissæ, the curves for the conductivities of Li, Na, Ca, and Ba gelatinate are almost identical (for the same pH), while the curves for the conductivities of K and NH_4 gelatinate are only little higher.

4. The curves for the viscosity and swelling of Ba (or Ca) and Na gelatinate are approximately parallel to those for osmotic pressure.

5. The practical identity or close proximity of the conductivities of metal gelatinates with univalent and bivalent metal excludes the possibility that the differences observed in the osmotic pressure, viscosity, and swelling between metal gelatinates with univalent and bivalent metal are determined by differences in the degree of ionization (and a possible hydratation of the protein ions).

6. Another, as yet tentative, explanation is suggested.

[Reprinted from The Journal of the American Medical Association, December 21, 1918, Vol. lxxi, No. 25, pp. 2050–2051.]

A SELECTIVE MEDIUM FOR B. INFLUENZAE.

Oleate-Hemoglobin Agar.

By O. T. AVERY,

Captain, M. C., U. S. Army.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

The reports of various observers indicate that great difference of opinion exists concerning the etiologic significance of *B. influenzae* in the present epidemic of influenza. Some investigators have been able to isolate this organism from a large percentage of the cases that they have observed; others have succeeded in recovering it in very few instances. It is possible that technical difficulties in the isolation and growth of this micro-organism may be in part responsible for the discordant results obtained in different laboratories. In any case, all those who have had experience in the isolation and cultivation of *B. influenzae* agree that in working with this organism, there are a considerable number of technical difficulties that have been only partly overcome by the methods so far devised.

In obtaining the organism from the sputum or throat cultures, or even from cultures made at necropsy, it is usually found in association with many other bacteria, most of which grow with greater luxuriance than does the influenza bacillus. It is evident, therefore, that if a medium could be devised that would prevent the growth of at least certain of these other organisms, such a medium would be of great assistance, especially if at the same time the growth of the influenza bacillus could be enhanced.

An attempt has been made to develop such a medium, and in the present report the results so far obtained are briefly described. In the development of this medium, use has been made of the observation that soaps of the unsaturated fatty acids are bactericidal for certain bacteria. In preliminary studies it has been found that the addition of sodium oleate to mediums prevents the growth of certain gram-positive organisms, principally pneumococcus and streptococ-

cus, while the growth of *B. influenzae* is enhanced by the presence of this substance. The oleate-hemoglobin medium, therefore, was devised and has proved very satisfactory for the isolation of this organism.

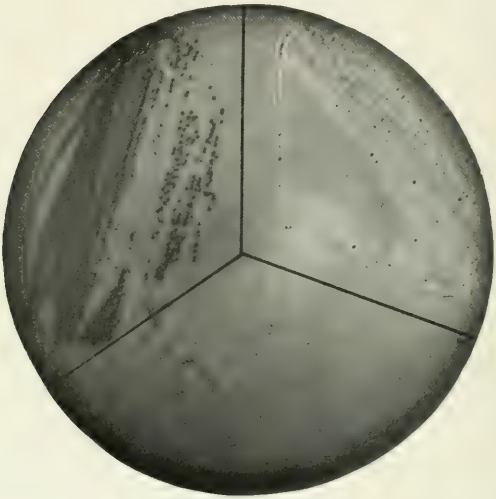


FIG. 1.—Plain blood agar plate: Lateral sectors show growth of pneumococcus and *Streptococcus hemolyticus*, lower sector *B. influenzae*, growth present but not visible in photograph.

Preparation of the Medium.

Meat infusion agar (2 per cent.), which is neutral or slightly alkaline in reaction, is used as a base. To this is added a solution of sodium oleate sufficient to make a final concentration of 1: 1,000. A serum-free suspension of red blood cells in broth is freshly prepared from sterile defibrinated rabbit's blood. One c.c. of this corpuscular suspension is added to each hundred c.c. of oleate agar, the addition of

blood being made while the medium is still hot. Plates are then poured containing about 15 c.c. each of oleate-hemoglobin agar and used fresh to avoid drying out of the medium.

In the preparation of oleate-hemoglobin agar attention should be given to certain details:

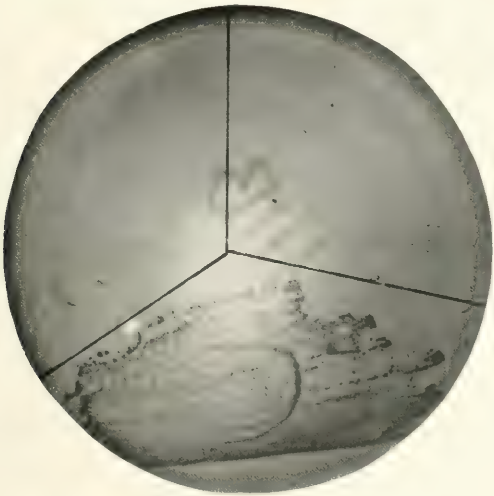


FIG. 2.—Oleate-hemoglobin agar plate: Corresponding sectors are planted with same organisms as in Figure 1 and show enhanced growth of *B. influenzae* in lower sector and complete inhibition of growth of pneum coccus and *Streptococcus hemolyticus*.

1. Agar. -Two per cent. meat infusion agar having a reaction of from 0.3 to 0.5 acid to phenolphthalein should be used. The initial hydrogen ion concentration of the agar should represent a *p*H of from 7.3 to 7.5. Hormone agar prepared according to the formula of Huntoon[1] yields excellent results, and has been used in the present study.

1. Huntoon, F. M.: Jour. Infect. Dis., 1918, 23, 169.

2. *Sodium Oleate.*—Two per cent. solution of sodium oleate (neutral) is made in distilled water, sterilized in the autoclave and kept as a stock solution. Five c.c. of this 2 per cent. solution of oleate is added to 95 c.c. of agar, giving a concentration of 1: 1,000. In the present work Kahlbaum's sodium oleate has been used, but other preparations are serviceable.

3. *Suspension of Red Blood Corpuscles.*—Sterile defibrinated rabbit's or human blood may be used. Since serum is known to inhibit the action of oleate, and since hemoglobin is the constituent of blood essential for growth of *B. influenzae*, a serum-free suspension of the red corpuscles is used instead of whole blood. The red cells are removed from the defibrinated blood by centrifugation, the supernatant serum is pipetted off, and the corpuscles are made up to the original volume of blood by the addition of broth. One c.c. of this suspension of red cells is added to each hundred c.c. of oleate agar. The suspension of blood corpuscles should be added directly while the medium is hot, and just before use.

4. *Formula.*—This calls for:

Meat infusion agar, 2 per cent.	94 c.c.
2 per cent. solution of sodium oleate	5 c.c.
Suspension of red blood cells	1 c.c.

Oleate-hemoglobin bouillon may be prepared in the same way by substituting broth in the place of agar in the foregoing formula.

Cultures taken from the nasopharynx by the West tube or from the throat by direct swabbing should be streaked on the surface of the oleate-hemoglobin medium according to the technic described for the detection of meningococcus carriers.[2] Similar plates should also be made from the sputum. At necropsy, cultures should be taken from scrapings of the tracheal and bronchial mucosa, as well as from the lung exudate.

All culture plates should be incubated for forty-eight hours at 37 C.

2. Standard Technic of Meningococcus Carrier Detection, Adopted by the Medical Departments of the United States Army and Navy, 1918.

Comment.

The use of this medium has led to an increase in the percentage of positive findings of *B. influenzae* in actual cases of the disease and in convalescents. A report of these studies will be made in a subsequent communication.

Because of more luxuriant growth on oleate-hemoglobin medium, the colony of *B. influenzae* appears larger and less translucent than on ordinary blood agar, and in a later stage of development is not infrequently nucleated. On this medium, the gram-negative cocci of the *M. catarrhalis* group, staphylococci and occasionally diphtheroid bacilli grow, while pneumococci and streptococci of the hemolytic and *S. viridans* variety fail to develop.

The use in culture mediums of soaps of the unsaturated fatty acids, of which sodium oleate is a representative, suggests the application of this principle in bacteriologic studies of other organisms.

[Reprinted from the AMERICAN JOURNAL OF DISEASES OF CHILDREN, January, 1919, Vol. xvii, No. 1, pp. 38–42.]

A METHOD FOR THE DETERMINATION OF FAT IN DRIED FECES AND ITS DISTRIBUTION AS SOAP, FREE FATTY ACIDS AND NEUTRAL FAT.

AN APPLICATION TO FECES OF THE ROESE-GOTTLIEB METHOD FOR DETERMINING FAT IN DRIED AND CONDENSED MILKS.

BY L. EMMETT HOLT, M.D., ANGELIA M. COURTNEY, AND HELEN L. FALES.

(*From the Laboratories of The Rockefeller Institute and the Babies' Hospital.*)

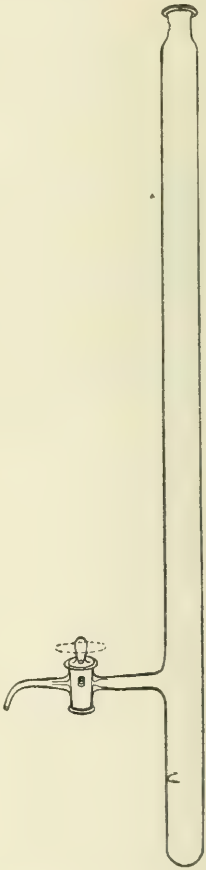
The investigation of the fat metabolism of infants and young children has been for some time an important subject of study in this laboratory. In furtherance of it, a large number of stools have recently been collected from the wards of the hospital, from other institutions and from various private patients. Careful data have been obtained regarding the food of the patients from whom stools were collected and so far as possible representative samples of the daily food were procured for analysis. Total fat has been determined in food and feces, and in the latter the distribution of the fat as soap, free fatty acids and neutral fat. In most cases total ash and calcium content of food and feces were also determined. The results of these investigations have been incorporated with many analyses which we had obtained in previous metabolism studies. It is our intention to present in a series of papers these data and to discuss their bearing on various problems of fat metabolism in infants and young children, especially the relation of the amount and the kind of the fat in the food to the quantity and distribution of the fat in the feces and the influence of fat on calcium metabolism.

In this preliminary paper we present the method used in obtaining the fat values. Previous to the autumn of 1915 a modification of the Soxhlet method was used for fat determination in this laboratory. This method has numerous disadvantages. It involves a forty-eight hour extraction with an elaborate equipment, which requires much attention. Moreover, in order to obtain complete extraction of the fat it is necessary to use very small quantities of material with a consequently high percentage error. The need of a shorter and simpler method led us to apply to the study of feces the Roese-Gottlieb method for the determination of fat in dried and condensed milks,

as practiced in the Dairy Laboratory, Bureau of Chemistry, Department of Agriculture.[1]

As adapted to the determination of total fat and fat distribution in feces, the method which we have employed is as follows:

Method.



One-gram samples of thoroughly dried and powdered material are weighed into small evaporating dishes. One half of these samples are used for the determination of total fat and the other half for the determination of the unsaponified fat; that is, the neutral fat and the free fatty acids. To each sample which is to be used for the determination of total fat there is added about 10 c.c. of dilute hydrochloric acid (one part of concentrated hydrochloric acid to three parts of water) and to those which are to be used for the determination of the unsaponified fat there is added the same volume of water; that is, about 10 c.c. The dishes are then placed on a water bath and left there until the contents are thoroughly heated and disintegrated. The homogeneous mixture from each dish is then transferred to a Röhrig tube (see illustration) and washed in, first with sufficient water to bring the contents of the tube exactly to a mark designating half the capacity of the portion below the faucet, then with 95 per cent. alcohol until the tube is filled to the level of the faucet. There is then added 25 c.c. of ethyl ether, free from nonvolatile matter, and the tube is shaken vigorously for a half minute; there is then added 25 c.c. of petroleum ether, boiling below 60°C., and the tube is again shaken for a half minute. The mixture is allowed to stand until the upper liquid is perfectly clear. The layer of ether, containing the extracted fat, is then drawn off by the faucet through a quick acting filter paper into a bottle of known weight. To make the extraction complete the process is repeated with 20 c.c. of both kinds of ether. For extreme accuracy a third extraction is made with 30 c.c. of the mixed ether, which has been recovered

1. Patrick: Roese-Gottlieb method. Circular 66, U. S. Dept. of Agric., Bureau of Chem.

from previous determinations. To insure against loss of fat, the tip of the faucet, the funnel and filter paper are washed with about 10 c.c. of mixed ether.

The ether is distilled from the combined extracts by means of an electric stove. The distillate, composed of mixed ethyl and petroleum ether, is condensed and used for further determinations. The fat residue is dried over night on a water bath, then for several hours in an oven at 98°C., desiccated and weighed.

In order to determine the free fatty acids the extract from the nonacidified samples is used. After the fat has been dried and weighed, it is dissolved in benzol, heated and titrated while very hot with tenth-normal alcoholic sodium hydrate. Phenolphthalein is used as an indicator and the titration is carried to the maximum color. The alkali used is standardized by stearic acid, dissolved in hot benzol.

The difference in weight between the extract from the acidified material (the total fat) and that from the nonacidified material (the neutral fat and free fatty acids) is the weight of soap fat. The difference between the weight of the nonacidified extract and the weight of the free fatty acids, computed from titration figures, represents the neutral fat. The following is an example:

	gm.
A. Weight of extract from acidified sample	0.38
B. Weight of extract from nonacidified sample	0.24
Difference between A and B = fat as soap	0.14
C. Titration of B..........6.0 c.c.	
Equivalent weight of free fatty acid	0.17
(1 c.c. tenth-normal NaOH = 0.0284 gm. stearic acid.)	
Difference between B and C = neutral fat	0.07

In the Roese-Gottlieb method ammonia is used to facilitate the separation of the fat from the protein. This step is necessarily omitted in the determination of fat in feces on account of the presence of the free fatty acids, which would be saponified by the ammonia.

The advantages of this method for fat determination are evident. The technic is simple, the amount of ether required for each determination is not large, that is, it can be distilled off quickly, and the results can be obtained in from eighteen to twenty-four hours from the weighing of the samples. Moreover, the method gives the distribution as soap fat, free fatty acids and neutral fat.

Considerable confusion exists in regard to the interpretation of reported figures for fat in feces because of the different methods which have been used. Two, which have been much quoted, the

Liebermann-Székely[2] and the Kumagawa-Suto[3] methods, give a value for total fat only, stated in terms of fatty acids. This is obtained by saponifying the fat, then splitting it with acid before extracting with alcohol and ether. The extract obtained is purified by reextracting with petroleum ether. The Kumagawa-Suto method gives higher values than most methods, because the extract is never heated in drying higher than 50 C. Thus the lower fatty acids are in part held in the extract. As the lower fatty acids arise mainly from the carbohydrate of the food, results which include them are not to be desired in a study of fat metabolism, especially since their separate determination is very difficult and doubtful.

The Gephart-Czonka[4] method involves the same principles as those mentioned, but the fatty acid value is obtained by titrating the petroleum ether extract instead of by weighing. This method requires considerable manipulation and the use of large quantities of ether. As in the methods just described, only total fat, as fatty acids, is obtained. These three methods avoid the slight objection which might be raised in regard to our own procedure, in which the total fat is weighed and reported as a mixture of fatty acids and neutral fat. This is not, however, of any practical importance, since the difference between the weight of the neutral fat and its corresponding fatty acid is so slight that the total fat value is not affected to the extent of 1 per cent. of the usual amounts weighed.

Folin and Wentworth[5] also obtain their figures in this form, that is, the total fat as a mixture of fatty acids and neutral fat, and they obtain a value for the neutral fat by titrating the fatty acids and subtracting the result from the total fat. They claim that there is no significance in a figure for soap fat as separate from fatty acids, since both in the intestines and during the drying of the feces on the water bath "numerous different factors of no particular bearing on fat metabolism will materially affect the equilibrium between the soap fat and the fatty acids." Our experience, however, in the study of infants' stools of various types has led us to the conclusion that in

2. Liebermann and Székely: Pflüger's Arch. f. d. ges. Physiol., **72:** 360, 1898.
3. Kumagawa and Suto: Biochem. Ztschr., **8:** 212, 1908.
4. Gephart and Csonka: Jour. Biol. Chem., **19:** 521, 1914.
5. Folin and Wentworth: Jour. Biol. Chem., **6:** 421, 1910.

spite of these objections the soap fat value, as determined, may be of significance in a study of fat metabolism. For this reason we feel strongly the advantage of a method by which it is possible to obtain this value.

In modifying the Roese-Gottlieb method so as to obtain the fat separation, use has been made of the Folin-Wentworth procedure for titrating the fatty acids. As a matter of convenience sodium hydrate dissolved in 95 per cent. alcohol and standardized by stearic acid is used instead of sodium alcoholate. The solution we use quickly becomes cloudy from contact with the glass container, so that frequent restandardization is necessary.

Various authors believe that the ether extract of acidified material includes substances other than fat, notably coloring matter. Ordinarily the extract which we obtain is clean, homogeneous, mainly crystalline and dissolves clear in any of the fat solvents. Occasionally there is an appearance of sediment in the extract and in some cases, especially in the stools of breast fed infants, the extract shows marked color. In such cases the dried extract is dissolved in mixed ether and again filtered before drying and weighing. If care is taken to prevent drawing off any of the watery portion from the tube with the ether, the need of filtering will be lessened. This is very desirable since slight mechanical loss in the second filtration is scarcely avoidable.

The completeness of extraction has been tested with samples of stearic acid, saponified stearic acid and mixtures of these. In every case the recovery was 99 per cent. or more. In order to make sure that the results do not include the acids formed from carbohydrate, weighed samples of stearic acid were dissolved in mixed ether, lactic and acetic acids added and the whole was evaporated on a water bath, dried and weighed. Invariably only the stearic acid remained.

It is not claimed that the method here described gives results with an average error less than 1 per cent. The fat forms so large a proportion of the dried feces that the homogeneity of the sample is of great importance. When the fat amounts to 50 per cent. of the dried weight of the feces, there may be variations of 5 per cent. in duplicate analysis. This extreme error is largely due to the fact

that stools with so large a proportion of fat are gummy and impossible to powder.

In some respects our method resembles that described by Saxon.[6] His method gives the distribution as fatty acids, soap fat and neutral fat and he extracts by shaking. His determination is made on moist fresh feces and he does not use mixed ethers. The use of the Röhrig tubes in the Roese-Gottlieb method greatly facilitates the manipulation.

6. Saxon: Jour. Biol. Chem., **17:** 99, 1914.

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THE USE OF THE FINAL HYDROGEN ION CONCENTRATION IN DIFFERENTIATION OF STREPTOCOCCUS HÆMOLYTICUS OF HUMAN AND BOVINE TYPES.

By OSWALD T. AVERY, M.D.,
Captain, Medical Corps, U. S. Army,

and GLENN E. CULLEN, Ph.D.,
Captain, Sanitary Corps, U. S. Army.

(From the Hospital of The Rockefeller Institute for Medical Research.)

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Streptococcus hæmolyticus is known to cause certain pathologic conditions in man, such as erysipelas, puerperal sepsis, cellulitis, septic sore throat, bronchopneumonia, etc. It is frequently associated with other diseases, often as a secondary invader in measles and scarlet fever. A hemolytic streptococcus of similar characteristics is also found as the etiologic agent in many diseases of lower animals, particularly in mastitis of cows. Furthermore, an organism of like character is almost constantly present in milk and dairy products. In milk-borne epidemics of septic sore throat the weight of evidence supports the view that the disease is caused by human types of hemolytic streptococci which are accidently communicated to the udder of the cow, rather than to the streptococci which are the common cause of bovine mastitis, and which are so frequently found in milk when no disease is present. From an epidemiological standpoint the recognition of distinctive human and bovine types of this organism is of considerable interest and practical importance.

The occurrence of a hemolytic streptococcus, resembling to such a degree the organism found in pathologic lesions of man, has made necessary the devising of methods for differentiation of this type of streptococcus from that of human origin.

The bacteriological diagnosis of these types rests upon the summation of a number of differential features, rather than upon the dis-

tinctive value of any one test. Minor morphological and cultural characters, differences in the thermal death-point, and variations in the degree of hemolytic activity and pathogenicity for rabbits serve to indicate that essential biologic differences exist between these two types. In addition, many observers have noted the fact that non-pathogenic hemolytic streptococci produce more acid in carbohydrate media than the pathogenic varieties. These observations have been based upon the titratable acidity recorded in terms of the amount of normal alkali required to neutralize a given amount of culture fluid.

Some work has been done on the final hydrogen ion concentration as a means of differentiation of these organisms. Clark (1), discussing the diagnostic value of the final hydrogen ion concentration in differentiating cultures of *Bacillus coli*, states: "Sufficient work has been done in this laboratory to indicate that the same phenomena . . . . apply to many of the streptococci." Ayers (2), studying 200 cultures of streptococci, found that two final zones of hydrogen ion concentration were reached: pH. 4.6 to 4.8, 5.5 to 6.0. Smillie (3) records five strains tested by this method, but does not lay stress upon the results obtained. Ayers, Johnson, and Davis (4) have published interesting observations on the limiting hydrogen ion concentration of a series of streptococci, and have found that in dextrose-yeast-peptone medium the strains from pathogenic sources have a lower limiting hydrogen ion concentration than that reached by the non-pathogenic forms. They emphasize again the fact that titratable acidity, as a measure of the fermentative activity of an organism, is influenced so much by the nature and buffer content of the culture medium that this method should be supplanted by the determination of the hydrogen ion concentration. Ayers[1] has suggested the use of the limiting hydrogen ion concentration in differentiation of human and bovine types of streptococci and has found that the organisms obtained from milk gave the high limiting hydrogen ion concentration, that is from pH 4.5 to 5.0, and that most of the cultures were hemolytic.

The present work is confined to a study of hemolytic streptococci, and from it there has developed the fact that the hydrogen ion con-

[1] Personal communication.

centration at which the human strains of *Streptococcus hæmolyticus* cease to grow is different from that which limits growth of hemolytic cultures from bovine sources.

EXPERIMENTAL.

Source of Cultures.—This study comprises 169 strains of *Streptococcus hæmolyticus*, of which 124 were of human origin and 45 from bovine sources. Among the latter are included a number of strains recovered directly from mastitis in cows and others isolated from milk. Two strains are included among these from bovine sources, which were isolated by Smith and Brown (5) from udders of cows during Boston epidemics of septic sore throat, and identified by them as of human type.[2] Nineteen strains came from cream cheese of different brands.

The strains of hemolytic streptococci of human origin were in the majority of instances isolated from the throats, sputum, blood, and lung lesions of men suffering from postmeasles bronchopneumonia, or from the throats of those intimately in contact with these patients during an epidemic of pneumonia at one of the camp hospitals. In addition, several strains of *Streptococcus hæmolyticus* from erysipelas, postpartum sepsis, meningitis, and cellulitis have been included.[3]

Methods of Isolation and Identification.—All the cultures were isolated and identified according to the standard methods for the isolation and identification of *Streptococcus hæmolyticus* adopted by the Medical Department of the United States Army. Isolation was made from a single colony on plain blood agar, and subsequent identification confirmed by the hemolysin test, the bile reaction, and the fermentation of certain test substances, as inulin, salicin, etc. The original stock cultures were preserved in plain blood agar stabs or plain blood broth.

[2] The authors acknowledge their indebtedness to Dr. J. Howard Brown and Dr. Frederick S. Jones, of the Department of Animal Pathology of The Rockefeller Institute, Princeton, N. J., for many of these cultures.

[3] Dr. Charles Krumwiede, of the Department of Health of the City of New York, has furnished many of these cultures, and the authors wish to express their appreciation of this courtesy.

Medium.—The medium used for the determination of the final hydrogen ion concentration was meat infusion broth with the addition of 1 per cent dextrose. This medium was prepared from the infusion of fresh beef and contained 1 per cent peptone, 0.5 per cent salt, and 1 per cent dextrose. The initial reaction varied from pH 7.6 to 7.8. With the exception of the dextrose, the medium was the same as that used in this laboratory for the routine cultivation of pneumococcus, and the method of preparation was identical with that described by Avery, Chickering, Cole, and Dochez (6).

In all the experiments measured quantities of the dextrose broth were inoculated with uniform amounts of an 18 hour plain broth culture of the organism to be tested and incubated at 37°C.

Determination of Hydrogen Ion Concentration.

The colorimetric or indicator method of determining the hydrogen ion concentration of bacterial cultures is, because of its simplicity and availability, the method of choice in studies of the fermentative activity of organisms. The principles and application of this method have been so thoroughly set forth by Michaelis (7) and by Clark and Lubs (8), as well as by other authors, that reference will be made only to the most important details. The color of the culture medium and the turbidity produced by the massive growth of organisms, as well as by precipitation of proteins, have been the principal sources of error. This changing or masking of the indicator color may be obviated in two ways. First, it must be remembered that all successful media are buffer solutions; that is, they contain salts that require relatively large changes in acid or base to produce small changes in the reaction. The hydrogen ion concentration of these solutions is not materially changed by the addition of water. It is possible, therefore, to dilute the color of the medium itself without affecting the reaction. Secondly, the color of the medium may be superimposed upon that of the indicator by the comparator method introduced by Walpole (9) and adopted by Clark and Lubs. The intense turbidity of cultures of *Streptococcus hæmolyticus* grown in dextrose broth may be reduced by use of the supernatant culture fluid, although the combination of the dilution and comparator methods is usually sufficient to insure an accuracy of pH $\pm$ 0.1.

The hydrogen ion concentration determinations were made as follows: Duplicate tubes containing 5 cc. each of the culture were diluted with redistilled water to 15 cc. One drop of a 0.1 per cent alcoholic solution of methyl red was then added to one tube while the other tube was used for the comparator color. The hydrogen ion concentration was then read in the comparator block, as modified by Dernby and Avery (10), and recorded to the nearest tenth of a pH. With this indicator the cultures with a reaction more acid than a pH of 4.5 showed a definite claret-red color, while those less acid than a pH of 5.0 showed only a slight salmon tint. This difference is striking. It is important to use the indicator in sufficiently low concentration. The final reaction of the more acid group of organisms is about the lower limit of usefulness of methyl red, that is pH of 4.3, and too much indicator makes it impossible to distinguish between 4.6 and 4.3. Our experience shows that one small drop, about 0.02 cc., gave the sharpest reading. If, however, four or five times as much methyl red is used, the difference in color tints within this narrow zone is much less distinctive. This point cannot be overemphasized, especially since there is a tendency for most workers to use an excess of indicator. The accuracy of the determinations in the range pH 4.6 to 4.0 was controlled in several experiments by the use of the indicator brom phenol blue (tetrabromophenolsulfonephthalein). The standard solutions of known hydrogen ion concentration were prepared for the range 5.8 to 8.0 from phosphates by Sörensen's technique, and for the range 4.0 to 5.8 from acetate by Walpole's directions. The phosphates had been previously standardized by the hydrogen electrode.

Influence of Dextrose.

Since Clark and Lubs (11), in using this method for differentiating the types of *Bacillus coli*, have shown that the amount of dextrose available for fermentation by the organism influenced the final reaction, and that if insufficient dextrose was used a reversion of reaction followed, it was necessary to determine the effect of varying concentrations of dextrose upon the final hydrogen ion concentration of *Streptococcus hæmolyticus*. Further, it was desired to determine whether or not the sugar present in the medium has any effect upon the reaction. The following experiment was therefore devised.

To plain broth of known hydrogen ion concentration, dextrose was added in quantities sufficient to make 0.5, 1, and 1.5 per cent dextrose concentration. The medium was incubated over night at 37°C. to test sterility and then inoculated with 0.1 cc. each of an 18 hour plain broth culture of *Streptococcus hæmolyticus*. Broth which had been fermented to remove muscle sugar was used in the same manner.[4] Samples containing 0.5, 1, and 1.5 per cent dextrose were

TABLE I.

Influence of the Concetrration of Dextrose on the Final Hydrogen Ion Concentration.

100 cc. bottles inoculated with 0.1 cc. each of 18 hour plain broth cultures of Human 24, Bovine C57, and Cheese 4.

Series No.	Culture medium.	Initial hydrogen ion concentration.	Hydrogen ion concentration.					
			24 hrs.			14 days.		
			Human.	Bovine.	Cheese.	Human.	Bovine.	Cheese.
		pH	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*
1	Plain broth.	7.6	7.35	7.25	7.15	7.3	7.1	7.3
2	" " + 0.5% dextrose.	7.5	5.0	4.5	4.5	5.1	4.5	4.5
3	" " + 1% "	7.4	5.1	4.5	4.3	5.1	4.5	4.3
4	" " + 1.5% "	7.5	5.0	4.5	4.3	5.1	4.5	4.3
5	Sugar-free broth + 0.5% dextrose.	7.5	5.1	4.5	4.3			
6	" " + 1% "	7.5	5.1	4.5	4.3	5.1	4.5	4.3
7	" " + 1.5% "	7.5	5.0	4.5	4.2	5.1	4.5	4.2

prepared and inoculated in the same way. The hydrogen ion concentration of the media, after the preliminary sterilization and incubation and before inoculation, was controlled, and 5 cc. samples of the supernatant liquid were removed from the bottles 24 hours and 2 weeks after inoculation (Table I). It is evident from these results that the concentration of dextrose from 0.5 to 1.5 per cent gives the same hydrogen ion concentration, and further, that either plain broth or sugar-free broth is equally available for this purpose. We have, therefore, used throughout the series an ordinary 1 per cent dextrose broth.

[4] In the preparation of sugar-free broth the meat infusion, before the addition of peptone, is fermented by *B. coli* for 18 to 24 hours.

Rate of Acid Production of Streptococcus hæmolyticus in 1 Per Cent Dextrose Broth.

It was important to determine the rate of acid production and length of time required to reach the final limiting hydrogen ion concentration. Hence the following experiments were carried out.

1 per cent dextrose broth was prepared from sugar-free medium, as in the previous experiment, and 100 cc. samples were inoculated with two human strains, two cheese strains, and one bovine strain. The cultures were incubated at 37°C., and at definite intervals 5 cc. duplicate samples were removed for examination. The results are given in Table II and plotted in Text-fig. 1.

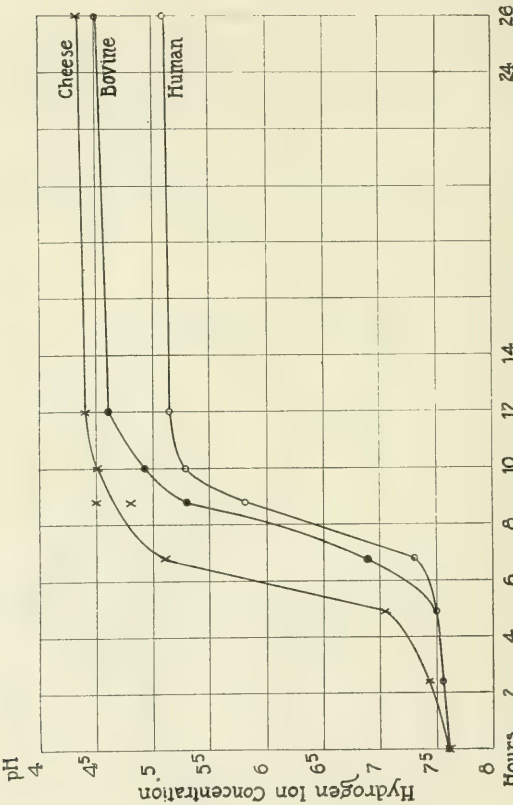
TABLE II.

Rate of Acid Production.

Sugar-free broth + 1 per cent dextrose. Initial pH = 7.6.

Strain No.	Hydrogen ion concentration.							
	2½ hrs.	4¾ hrs.	6¾ hrs.	9 hrs.	10 hrs.	12 hrs.	26 hrs.	15 days.
	pH	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*
Human 24	7.55	7.5	7.3	5.9	5.3	5.15	5.1	
" 43	7.5	7.5	7.3	5.7	5.25	5.15	5.1	
Cheese 64	7.45	7.05	5.1	4.8	4.5	4.3	4.3	4.3
" 2	7.45	7.1	5.1	4.5	4.5	4.4	4.4	
Bovine C57	7.5	7.5	6.9	5.3	4.95	4.6	4.5	4.4

It is evident from these results that the organisms from all three sources reached their final hydrogen ion concentration within 24 hours. The values for the duplicate cultures were so close that only one curve for each set is apparent. Although the curve for acid production of these organisms had reached the maximum within 24 hours, it seemed desirable to determine whether or not continued incubation produced any change in the hydrogen ion concentration. For this purpose nine strains were inoculated in 100 cc. portions of 1 per cent dextrose broth and incubated at 37°C. (Table III). These results substantiate the preceding experiments in that the final hydrogen ion concentration is reached within 24 hours, and show further that this final hydrogen ion concentration does not change on continued incubation. Although 14 days was the longest period



TEXT-FIG. 1. Rate of acid production of human and bovine types of *Streptococcus hæmolyticus*.

of test in this series, the same results have been obtained from cultures tested after 3 weeks.

TABLE III.

Effect of Prolonged Incubation.

100 cc. of sugar-free broth + 1 per cent dextrose inoculated with 0.05 cc. of 18 hour plain broth cultures. Initial pH = 7.3.

Strain No.	Source.	Hydrogen ion concentration.				
		18 hrs.	2 days.	4 days.	7 days.	14 days.
		pH	*pH*	*pH*	*pH*	*pH*
24	Human.	5.2	5.1	5.1	5.1	5.1
66	"	5.2	5.2	5.2	5.1	
2	"	5.1	5.1	5.1	5.1	
43	"	5.3	5.1	5.1	5.1	
14	"	5.2	5.1	5.15	5.1	
118	"	5.2	5.2	5.1	5.1	
276	"	5.2	5.1	5.1	5.1	
277	"	5.2	5.1	5.1	5.1	5.1
11	"	5.15	5.1	5.1	5.1	5.1

Effect of Animal Passage upon the Final Hydrogen Ion Concentration.

Since the question of virulence is always of first importance in a discussion of the biology of an organism, it was deemed advisable to determine whether or not animal passages had any effect upon the final hydrogen ion concentration. To determine this five original cultures and their corresponding subcultures, which had been passed through a number of animals, were selected and inoculated in 100 cc. portions of 1 per cent dextrose broth. The results are given in Table IV. It is evident from this experiment that the final hydrogen ion concentration of pathogenic *Streptococcus hæmolyticus* is not affected by animal passage.

Constancy of the Final Hydrogen Ion Concentration of the Same Strains in Different Experiments.

The constancy of the final hydrogen ion concentration of *Streptococcus hæmolyticus* is strikingly shown in Table V in which the hydrogen ion concentration of the same strains, but of different experiments, have been assembled.

TABLE IV.

Influence of Animal Passage on the Final Hydrogen Ion Concentration.

Strain No.	No. of animal passages.	Hydrogen ion concentration.		
		24 hrs.	48 hrs.	8 days.
		pH	*pH*	*pH*
1		5.1	5.1	5.1
1	7	5.3	5.3	5.3
23		5.1	5.1	5.1
23	16	5.15	5.1	
276		5.15	5.1	5.1
276	25	5.1	5.1	5.1
24		5.3	5.15	5.1
24	25	5.1	5.1	5.1
84		5.1	5.1	5.1
84	16	5.3	5.3	5.3

TABLE V.

Constancy of the Final Hydrogen Ion Concentration of the Same Strains in Different Experiments.

1 per cent dextrose in all media.

Experiment No.	Strain No.					
	Human.		Bovine.		Cheese.	
	24	8	C53	C57	4	I
	pH	*pH*	*pH*	*pH*	*pH*	*pH*
1	5.1					
2		5.2				
3			4.4	4.4	4.3	4.3
4, *a*	5.15	5.1				
4, *b*	5.1	5.3				
5			4.3	4.3	4.3	4.3
6	5.1			4.5	4.3	4.3
7			4.5	4.5		
8	5.1			4.5	4.3	
9	5.1			4.5	4.3	
10					4.5	

TABLE VI.

Final Hydrogen Ion Concentration of Streptococcus hæmolyticus of Known Human Origin.

Strain No.	Source.	Diagnosis.	pH
1	Autopsy (lung).	Bronchopneumonia following measles.	5.2
5	" "	" " "	5.1
20	" "	" " "	5.1
29	Sputum.	" " "	5.15
32	Autopsy (lung).	" " "	5.0
39	" "	" " "	5.2
59	" (blood).	" " "	5.1
78		" " "	5.2
92	Pleural fluid.	" " "	5.2
93		" " "	5.1
97	Pleural fluid.	" " "	5.1
107	Sputum.	" " "	5.3
110		" " "	5.1
113		" " "	5.1
118	Pleural fluid.	" " "	5.2
136		" " "	5.0
137	Throat.	" " "	5.1
138	Sputum.	" " "	5.1
142		" " "	5.1
145	Throat.	" " "	5.1
149	Blood.	" " "	5.1
151	Pleural fluid.	" " "	5.1
49	Sputum.	" " German measles.	5.1
2	Autopsy (lung).	"	5.1
3	" "	"	5.1
14	" "	"	5.1
24	" "	"	5.1
50	Pleural fluid.	"	5.1
55	" "	"	5.0
67	Blood.	"	5.1
72	Throat.	"	5.1
84	Pleural fluid.	"	5.2
139	Throat.	"	5.1
143	Pleural fluid.	"	5.2
152	" "	"	5.1

TABLE VI—*Continued.*

Strain No.	Source.	Diagnosis.	pH
10	Throat.	Measles.	5.1
37	"	"	5.0
43	"	"	5.1
47	"	"	5.1
53	"	"	5.1
60	"	"	5.1
64	"	"	5.1
66	"	"	5.2
71	"	"	5.1
79	"	"	5.1
82	"	"	5.0
83	"	"	5.1
85	"	"	5.1
86	"	"	5.1
88	"	"	5.1
89	"	"	5.1
96	"	"	5.2
98	"	"	5.2
108	"	"	5.1
116	"	"	5.2
117	"	"	5.1
119	"	"	5.0
126	"	"	5.2
127	"	"	5.1
128	"	"	5.1
141	"	"	5.1
148	"	"	5.15
150	"	"	5.1
4	"	German measles.	5.0
28	"	" "	5.1
38	"	" "	5.0
45	"	" "	5.1
47	"	" "	5.1
48	"	" "	5.1
54	Sputum.	" "	5.0
70	Throat.	" "	5.1
115	"	" "	5.1
134	"	" "	5.1
140	"	" "	5.1

TABLE VI—*Continued.*

Strain No.	Source.	Diagnosis	pH
9		Lobar pneumonia.	5.1
11	Sputum.	" " (Type I).	5.1
15	Throat.	" "	5.1
16	Sputum.	" "	5.2
27	Autopsy (lung).	" "	5.1
44	Throat.	" "	5.1
56	Autopsy (lung).	" "	5.1
61	Sputum.	" " (Type IV).	5.2
62	Throat.	" "	5.1
65	Sputum.	" "	5.1
69	Autopsy (lung).	" "	5.0
75		" "	5.1
87	Sputum.	" "	5.2
95	Throat.	" "	5.1
99		" "	5.1
120	Throat.	" "	5.1
125	"	" "	5.1
133		" "	5.1
144	Throat.	" "	5.1
8	"	Pneumonia.	5.1
281	Sputum.	"	5.1
286	Pleural fluid.	"	5.1
6	Throat.	Incipient tuberculosis.	5.1
34	"	" "	5.1
41	"	" "	5.1
46	"	" "	5.1
121	"	" "	5.2
122	"	" "	5.1
129	"	" "	5.1
40	Pericardial fluid.		5.0
267	Foot.	Cellulitis.	5.0
271	Blood.	Septicemia.	5.0
276	Pus.	Pelvic abscess.	5.1
277	" (abdomen).		5.1
264	Blood.	Osteomyelitis.	5.2
266	"	Meningitis.	5.0
306	Spinal fluid.	"	5.0

TABLE VI—*Concluded.*

Strain No.	Source.	Diagnosis.	pH
SH	1913 Boston epidemic.	Peritonitis.	5.0
S8	1917 " "	Empyema.	5.2
38	1912 Baltimore "	Adenitis.	5.0
C64B	Throat.		5.1
C63B	"		5.1
57*	"	Normal.	4.8
91	"	Measles.	4.8
103	"	"	4.9
105	"	Influenza.	4.9
288	Sputum.	Pneumonia.	4.8
X35B		Erysipelas.	4.9
A103B	Throat.		4.8
30	1912 Chicago epidemic.	Peritonitis.	4.9

* The eight human strains with a pH of 4.9 and 4.8 have been grouped together without reference to diagnosis.

TABLE VII.

Final Hydrogen Ion Concentration of Streptococcus hæmolyticus from Cheese.

Strain No.	pH	Strain No.	pH
Ch. 1	4.3	Ch. 11	4.4
" 2	4.4	" 12	4.5
" 3	4.3	" 13	4.4
" 4	4.4	" 14	4.4
" 5	4.4	" 15	4.5
" 6	4.3	" 16	4.3
" 7	4.5	" 17	4.5
" 8	4.3	" 18	4.3
" 9	4.5	" 19	4.5
" 10	4.5		

Survey of Streptococcus hæmolyticus.

With the assurance given that the media and conditions of growth were suitable for a survey of streptococci from various sources, we have completed the examination of 124 strains of *Streptococcus hæmolyticus* of known human origin and 45 from bovine sources, including 19 cultures of hemolytic streptococci isolated from cheese and 26 from milk. These results have been assembled in Tables VI to VIII, and are represented graphically in Text-fig. 2.

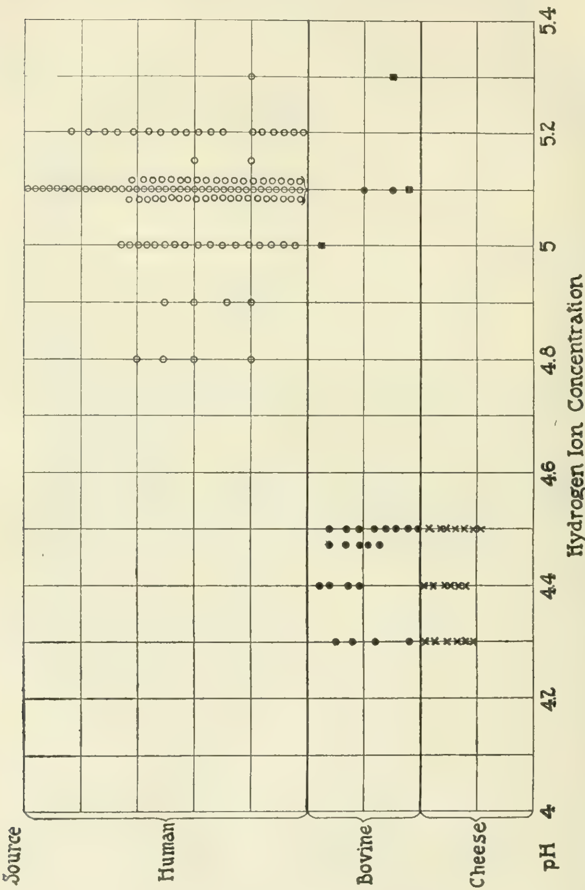
TABLE VIII.

Final Hydrogen Ion Concentration of Streptococcus hæmolyticus from Bovine Sources.

Strain No.	Source.	pH	Strain No.	Source.	pH
C67	Mastitis.	4.5	M2	Milk.	4.3
C57	"	4.5	M41	"	4.5
C53	"	4.5	M43	"	4.5
C69	"	4.5	V1	Udder.	4.4
C59	"	4.3	V2	"	4.3
M26	"	4.5	V3*	"	5.0
M93	Milk.	4.5	V4	"	4.3
M53*	"	5.3	V5	"	4.5
M3	"	4.5	V6	Milk.	4.5
MJ1*	"	5.1	V7	Udder.	4.4
M65	"	5.1	V8	"	4.5
M86*	"	4.5	V9	"	4.5
M1	"	4.4	V10*	"	5.1

* Strains V3 and V10 were isolated by Smith and Brown (5) from the udders of cows during Boston epidemics of septic sore throat and identified by them as human types. Strains MJ1, M86, and M53 were recovered by Jones from milk of cows showing no evidence of mastitis, and were described by him as either human strains which had contaminated the milk or streptococci from the skin or feces of cows. None of these cultures has been tested for pathogenicity. He found that none of the three strains agglutinated in the immune serum prepared by immunization of animals with *Streptococcus hæmolyticus* from bovine mastitis (12).

The results from this study are striking. None of the cheese strains shows a final hydrogen ion concentration less than a pH of 4.5. Of the 26 cultures from bovine sources, 21 had a final reaction between 4.3 and 4.5. Five showed a reaction of from 5.0 to 5.3; of these 5, 2 were received from Dr. J. H. Brown, and were designated by him, from their cultural characteristics, as human streptococci. The other 3 are of questionable diagnosis (foot-note, Table VIII). Of the 124 strains from known human origin, only 8 showed a pH less than 5.0 and none less than 4.8. It must be emphasized that the final hydrogen ion concentration of none of the large number of organisms examined occurred within the zone of 4.5 to 4.8, and only 8 in the zone of 4.8 to 5.0. This is important, for the zone below a pH of 4.5 showed a distinct red color with methyl red, while a reaction above 5.0 showed only a very faint salmon pink.



TEXT-FIG. 2. Difference in final hydrogen ion concentration of human and bovine types of *Streptococcus hæmolyticus*.

Practical Application of the Method of Hydrogen Ion Concentration in the Differentiation of Human and Bovine Types of Streptococcus hæmolyticus.

The difference in the final hydrogen ion concentration of these two types of organisms has been expressed in terms of the pH values throughout the present discussion. In the practical application of this method, however, it is not necessary to determine the actual final hydrogen ion concentration. Methyl red reacts in such a way at the different final hydrogen ion concentrations reached by human and bovine strains, that no difficulty is experienced in judging these color values directly. At a pH value of 5.0 to 5.2, representing the range of the final hydrogen ion concentration of human strains, the color of this indicator is a faint salmon pink, whereas at a pH of 4.3 to 4.5, the final value for organisms of bovine origin, the color is a decided red. This color difference is so marked that comparison with standard solutions is not necessary and slight experience suffices to permit the determination of the type from the color produced by the direct addition of methyl red to the culture.

In determining the type *Streptococcus hæmolyticus* the following procedure has been adopted. The strain to be tested is grown in test-tubes (7 by $\frac{7}{8}$ inches) containing 5 cc. of 1 per cent dextrose broth. After the maximum growth has been reached, generally within 24 to 48 hours depending upon the size of the inoculum and the suitability of the medium, the culture fluid is diluted with 10 cc. of distilled water, and one drop of 0.1 per cent alcoholic solution of methyl red added. The difference in color, representing the pH values, distinctive for the human and bovine types of *Streptococcus hæmolyticus* is immediately apparent. This reaction is sufficiently constant to be relied upon, and although an occasional atypical strain may be encountered, it constitutes a presumptive test of real value in differential diagnosis.

DISCUSSION.

The bacteriological methods used at present in the differentiation of human and bovine types of *Streptococcus hæmolyticus*, namely the

determination of their hemolytic and fermentative activity, and pathogenicity for rabbits, are time-consuming and inadequate. In addition, the determination of the titratable acidity produced by these organisms in the fermentation of certain test substances is admittedly unreliable. The corelation of these several reactions, however, together with minor morphological and cultural characters indicates clearly that distinctive differences of a biologic nature exist between hemolytic streptococci found in pathologic lesions of man and those occurring in bovine mastitis, in milk and dairy products. Any method, therefore, for the rapid differentiation of these closely allied organisms would be of inestimable advantage in epidemiological investigation.

From the data recorded in these experiments it appears that the final hydrogen ion concentration of human and bovine hemolytic streptococci is a constant and distinctive characteristic of these organisms. The human type of *Streptococcus hæmolyticus* reaches a final hydrogen ion concentration of pH 5.2 to 5.0, and the bovine type of pH 4.5 to 4.3. It has been found that in the medium described, the concentration of dextrose, 0.5 to 1.5 per cent, does not affect the final hydrogen ion concentration. Acid production in dextrose medium proceeds rapidly and the maximum acidity is reached in 24 to 48 hours, depending on the size of the inoculum and suitability of the medium for growth. This final reaction inhibits growth and does not change on further incubation of the culture. It also appears from these experiments that the final hydrogen ion concentration of *Streptococcus hæmolyticus* is not related to virulence, since no variation was found in the reaction of cultures as originally isolated and of the same cultures after repeated animal passage.

All the cultures studied were of known origin, and on the basis of clinical evidence and bacteriological methods had been previously identified as belonging to one or the other of these two types. By the method described 95 per cent of all the strains examined were accurately and rapidly classified by the determination of their final hydrogen ion concentration in 1 per cent dextrose broth.

The final hydrogen ion concentration of the strains from bovine sources has in no instance fallen in the intermediary zone between the limits distinctive for human and bovine types. Of the five strains of bovine origin giving the same final hydrogen ion concentration

as human strains, two had previously been identified by other methods as belonging to the human type. The other three may be either human strains which have contaminated the milk or streptococci from the skin or feces of cows (foot-note, Table VIII). Further investigation should be made on the final hydrogen ion concentration of hemolytic streptococci from feces and skin of cows.

In the practical application of these facts use has been made of the difference in color of methyl red at the final hydrogen ion concentration of these two types. This change in color is sufficiently distinctive in itself to serve as a direct test for type determination and obviates the necessity of comparative readings with accurately standardized solutions of the phosphates. Because of the accuracy and constancy of the final hydrogen ion concentration, this method offers a presumptive test of distinct value in the classification of hemolytic streptococci.

SUMMARY.

1. Under the conditions of these experiments, there appears to be a distinct and constant difference in the final hydrogen ion concentration of *Streptococcus hæmolyticus* from human and bovine sources.

2. Of 124 strains of *Streptococcus hæmolyticus* from known human origin, 116 reached a final hydrogen ion concentration of from pH 5.0 to 5.3. Only 8 reached a pH more acid than 5.0 and none more acid than pH 4.8.

3. Of 45 strains of *Streptococcus hæmolyticus* from bovine sources, including 26 strains isolated from milk and the udder of cows, and 19 from cream cheese, 40 reached a final hydrogen ion concentration of pH 4.3 to 4.5. Of the remaining 5 which reached a pH of 5.0 to 5.2, two were of known human type and three of uncertain diagnosis.

4. A rapid and practical application of this method is proposed as a presumptive test in the differentiation of human and bovine types of *Streptococcus hæmolyticus*.

BIBLIOGRAPHY.

1. Clark, W. M., *J. Biol. Chem.*, 1915, xxii, 87.
2. Ayers, S. H., *J. Bacteriol.*, 1916, i, 84.
3. Smillie, W. G., *J. Infect. Dis.*, 1917, xx, 45.
4. Ayers, S. H., Johnson, W. T., Jr., and Davis, B. J., *J. Infect. Dis.*, 1918, xxiii, 290.
5. Smith, T., and Brown, J. H., *J. Med. Research*, 1914–15, xxxi, 455.
6. Avery, O. T., Chickering, H. T., Cole, R., and Dochez, A. R., Acute lobar pneumonia. Prevention and serum treatment, Monograph of The Rockefeller Institute for Medical Research, No. 7, New York, 1917.
7. Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.
8. Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1.
9. Walpole, G. S., *Biochem. J.*, 1911, v, 207.
10. Dernby, K. G., and Avery, O. T., *J. Exp. Med.*, 1918, xxviii, 345.
11. Clark, W. M., and Lubs, H. A., *J. Infect. Dis.*, 1915, xvii, 160; *J. Biol. Chem.*, 1917, xxx, 209.
12. Jones, F. S., *J. Exp. Med.*, 1918, xxviii, 253.

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COMPARISON OF THE CARBON DIOXID TENSION OF THE ALVEOLAR AIR AND THE HYDROGEN-ION CONCENTRATION OF THE URINE WITH THE BICARBONATE OF THE BLOOD PLASMA.*

By OSCAR M. SCHLOSS, M.D., and HELEN HARRINGTON, M.A.

During the past two years data have been obtained from normal infants and from infants suffering from acidosis which permit a comparison of the carbon dioxid tension of the alveolar air, and the acidity of the urine with the plasma bicarbonate.[1]

Technic.

Alveolar air was collected by the Plesch-Higgins method as modified by Howland and Marriott. A large number of the analyses were made by the Haldane apparatus, the remainder by the colorimetric method of Marriott.[2] We have found the latter method to be quite accurate when carefully used and the results vary but little from those obtained by the Haldane apparatus. It seemed evident that any errors inherent in the colorimetric method were less than those associated with the method of collection of the alveolar air.

The plasma bicarbonate was determined by the method of Van Slyke and Cullen;[3] corrections for temperature and barometric pressure were made from their table.

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* From the Children's Wards, Bellevue Hospital, Dr. L. E. La Fétra, Director, and the Pathological Laboratory, Bellevue Hospital, Dr. Douglas Symmers, Acting Director. This work was aided by a grant from The Rockefeller Institute for Medical Research.

1. All cases of acidosis considered in this paper were of the variety due to gastro-enteric disorders.

2. Marriott, J. McK.: J. A. M. A. **66:** 1594, 1916.

3. Van Slyke, D. D., and Cullen, G. E.: J. Biol. Chem. **30:** 289, 1917, their table.

Hydrogen-ion concentration of the urine was determined by the method of Henderson and Palmer.[4]

When the alveolar air and the plasma bicarbonate were compared, the alveolar air was collected first and the blood withdrawn within a few minutes. When crying occurred during the air collection the infant was quieted and kept so for a few minutes before the blood was taken. The blood was withdrawn from the superior longitudinal sinus by means of a syringe, care being taken to prevent the entrance of air. By means of a glass tube and rubber attachment applied directly to the syringe, the blood was transferred underneath a layer of mineral oil in a centrifuge tube, contact with the air thus being entirely avoided. By these precautions the values obtained were for venous blood and could therefore accurately be compared with the carbon dioxid tension of alveolar air collected by the Plesch-Higgins method.[5]

Comparison of the hydrogen-ion concentration of the urine and the plasma bicarbonate was effected as follows: The infant was placed on a metabolism frame and the urine collected for four hours. At the end of two hours, blood was withdrawn for the determination of the plasma bicarbonate.

Comparison of Plasma Bicarbonate with Carbon Dioxid Tension of Alveolar Air.

Normal Infants.—The plasma bicarbonate of normal infants[6] ranged from 49 to 72 volumes per cent. of carbon dioxid bound as bicarbonate at 0° and 760 mm. barometric pressure. These values are lower than those for normal adults.[7] The carbon dioxid tension of alveolar air varied from 34 to 45 mm. of mercury. The carbon dioxid tension of alveolar air and the plasma bicarbonate of normal infants are shown in Table 1.

$$\frac{\text{The plasma bicarbonate } (Co_2,\ \text{V. P. C.})}{Co_2 \text{ tension of alveolar air}}$$

ranges from 1.22 to 1.84 with an average value of 1.5. The factor by which the carbon dioxid of the plasma bound as bicarbonate must be multiplied to convert it into terms of carbon dioxid tension of alveolar

4. Henderson, L. J., and Palmer, W. W.: J. Biol. Chem. **17**: 305, 1914.

5. This method gives the carbon dioxid tension of venous blood.

6. All of the patients on whom investigations were conducted were under 18 months of age.

7. Van Slyke, Stillman, and Cullen: J. Biol. Chem. **30**: 401, 1917. Gettler and Baker, J. Biol. Chem. **25**: 211, 1916.

air is $\frac{1}{1.5} = 0.66$. The close correspondence between the direct determination of the carbon dioxid tension of alveolar air and its calculation from the plasma bicarbonate is shown in Table 1. It is of considerable interest that the factors 1.5 and 0.66 are identical with those which apply to adults.[7] There has always been some question

TABLE 1.

Comparison of the Tension of Carbon Dioxid in Alveolar Air with the Plasma Bicarbonate in Normal Infants.

Case No.	CO_2 Tension of Alveolar Air, Mm.	Plasma Bicarbonate. CO_2 Bound at 0° and 760 Mm., Volume per Cent.	Plasma Bicarbonate. Volume per Cent. of CO_2 Bound at 0° and 760 Mm. CO_2 Tension of Alveolar Air, Mm.	Plasma Bicarbonate. (Volume per Cent. CO_2) $\times$ 0.66 to Give CO_2 Tension of Alveolar Air, Mm.
1	38.0	61.7	1.62	40.7
2	35.0	52.1	1.49	33.9
3	37.5	52.1	1.39	34.4
4	34.0	59.5	1.75	39.2
5	34.0	49.8	1.46	32.9
6	37.0	55.6	1.50	36.8
7	40.0	49.0	1.22	32.4
8	45.0	71.0	1.58	46.8
9	38.0	54.0	1.42	35.6
10	39.0	59.7	1.61	39.4
11	38.0	52.2	1.37	33.9
12	32.5	51.8	1.60	34.2
13	38.0	59.7	1.57	39.4
14	40.0	61.3	1.53	40.5
15	41.5	60.4	1.45	39.9
16	39.8	61.9	1.55	40.9
17	36.3	51.2	1.41	33.9
18	44.1	73.2	1.65	48.2
19	40.6	60.0	1.48	39.6
20	39.0	71.7	1.84	47.3
21	43.2	58.6	1.38	38.6

as to the accuracy of the collection of alveolar air from infants. The fact that the air is collected for a definite time, despite the rapidity and amplitude of respiration, suggests a possible source of error. Our results, however, indicate that in normal infants the collection of alveolar air by the Plesch-Higgins method as modified by Howland and Marriott gives an accurate index of the plasma bicarbonate.

Infants with Small Tidal Air Volume.—The accuracy of the collection of a sample of alveolar air from infants is dependent to some degree on the manner in which the infant breathes. This fact has been pointed out by Marriott. With a little experience one can tell almost exactly whether the air collection is satisfactory. In very small infants whose tidal air volume is small, or sick infants with shallow respirations, the analysis of alveolar air may be very misleading. The results from a group of such infants are shown in Table 2.

TABLE 2.

Comparison of Tension of Carbon Dioxid in Alveolar Air with Plasma Bicarbonate in Infants with Shallow Respiration.

Case No.	CO_2 Tension of Alveolar Air, Mm.	Plasma Bicarbonate. CO_2 Bound at 0° and 760 Mm., Volume per Cent.	Plasma Bicarbonate (Volume per Cent. CO_2) × 0.66 to Give CO_2 Tension of Alveolar Air, Mm.	Difference Between Determined CO_2 Tension of Alveolar Air and CO_2 Tension of Alveolar Air Calculated from Plasma Bicarbonate
1	24	51.3	33.8	9.8
2	25	....	45.5	20.5
3	30	63.5	42.0	12.0
4	22	54.0	35.3	13.3
5	16	48.0	31.3	15.3
6	21	53.0	35.0	14.0
7	26	62.0	41.5	36.0
8	27	58.0	38.0	11.0
9	22	56.0	37.0	15.0

It is quite evident that a consideration of carbon dioxid tension of alveolar air alone would have led to the diagnosis of acidosis, while the plasma bicarbonate showed that none existed. The reason for this error is obvious. The tidal air volume is so small that but little air enters the bag, most being taken up by the dead space in the mask. The air withdrawn from the bag for analysis contains a large proportion of atmospheric air. In practically every case shown in Table 2 it was realized at the time the test was made that the collection of alveolar air was unsatisfactory, and that the results would be too low.

From these results it seems evident that the carbon dioxid tension of alveolar air and the plasma bicarbonate correspond closely only in infants whose tidal air is of moderate volume. In infants with shallow

respirations, the alveolar air may lead to the diagnosis of acidosis when none exists. In such cases the plasma bicarbonate must be used as a guide or a mask devised which has a smaller dead space.

Infants Suffering from Acidosis.—The results from cases of acidosis are given in Table 3. They show a fairly close correspondence between the alveolar air and the plasma bicarbonate. This, however, is true only before the administration of sodium bicarbonate. In Table 4 are shown results from a group of infants who had been given a sufficient amount of sodium bicarbonate to restore the alkaline reserve. In many of the cases the alveolar air analysis showed results which were too low despite the correction of acidosis. It seems that the respiratory center may remain hyperirritable after the correction of acidosis. Whether this is a direct result of the disease, or whether the respiratory center is capable of stimulation by conditions other than an increased hydrogen-ion concentration of blood, remains to be determined.

Comparison of the Hydrogen-Ion Concentration of the Urine and the Plasma Bicarbonate.

Effect of Food.—In Tables 5, 6 and 7 are shown the hydrogen-ion concentration of the urine (negative logarithm) and the plasma bicarbonate of apparently normal infants who were receiving different types of food. The hydrogen-ion concentration ranged from a maximum of 4.1 to a minimum of 6.9. The average hydrogen-ion concentration of the urine from all cases was 5.24. Henderson and Palmer found that the urine of adults showed an average hydrogen-ion concentration of 6. The hydrogen-ion concentration of the urine of infants closely corresponds to the lower carbon dioxid tension of alveolar air and the lower plasma bicarbonate.

Marriott suggests that the lower alveolar carbon dioxid in infants is due probably to more active metabolism, leading to the greater production of acids. Tables 5, 6 and 7 show that the hydrogen-ion concentration of the urine bears a definite relationship to the type of food. The urine was more acid in infants receiving food richer in protein. The tables of Gettler show that the ash of milk is very weak in bases and could therefore neutralize but little acid.[8]

8. Gettler, A. O.: Dissertation, Columbia University, 1912.

TABLE 3.

Comparison of Tension of Carbon Dioxid in Alveolar Air with Plasma Bicarbonate in Infants with Acidosis.

Case No.	CO_2 Tension of Alveolar Air, Mm.	Plasma Bicarbonate. CO_2 Bound at 0° and 760 Mm., Volume per Cent.	Plasma Bicarbonate. (Volume per Cent. CO_2) × 0.66 to Give CO_2 Tension of Alveolar Air, Mm.	Difference Between Determined CO_2 Tension of Alveolar Air and CO_2 Tension of Alveolar Air Calculated from Plasma Bicarbonate
1	14	32.6	21.8	7.8
2	23	37.3	24.6	1.6
3	28	39.1	25.8	2.2
4	17	24.3	15.0	2.0
5	19	22.3	14.6	4.3
6	22	21.4	14.1	7.9
7	26	37.3	24.6	1.4
8	20	41.1	27.1	7.1
9	17	25.4	16.75	0.25
10	26	44.8	29.6	3.6
11	23	39.1	25.8	2.8
12	20	36.0	23.8	3.8
13	26	48.1	31.7	5.7
14	31	41.6	27.4	3.6
15	22	35.4	23.4	1.4
16	28	45.7	30.1	2.1
17	20	22.3	14.7	5.3
18	26	43.0	28.4	2.4
19	26	48.4	31.9	5.9
20	24	42.4	27.3	3.3
21	25	44.3	29.2	4.2
22	11	14.4	9.5	1.5
23	22	37.0	14.5	7.5
24	18	19.7	13.0	5.0
25	19	39.1	25.8	6.8
26	28	33.0	21.8	6.2
27	27	38.5	25.4	2.4
28	28	42.4	28.0	0.0
29	33	50.1	33.1	0.1
30	18	26.4	17.4	0.6
31	20	39.6	26.2	6.2
32	24	36.0	23.8	0.2
33	20	30.9	20.4	0.4

TABLE 4.

Comparison of Carbon Dioxid Tension of Alveolar Air with Plasma Bicarbonate in Cases of Acidosis after Correction of the Acidosis.

Case No.	CO_2 Tension of Alveolar Air, Mm.	Plasma Bicarbonate. CO_2 Bound at 0° and 760 Mm., Volume per Cent.	Plasma Bicarbonate. (Volume per Cent. CO_2) $\times$ 0.66 to give CO_2 Tension of Alveolar Air, Mm.	Difference Between Determined CO_2 Tension of Alveolar Air and CO_2 Tension of Alveolar Air Calculated from Plasma Bicarbonate	Sodium Bicarbonate, Gm.
2 (a)	23	37.3	24.6	1.6	6.0 subcutaneously
(b)	29	55.0	36.1	7.1	
5 (a)	17	24.3	15.0	2.0	5.4 subcutaneously
(b)	23	57.2	37.1	14.1	
14 (a)	23	39.1	25.8	2.8	7.0 intravenously
(b)	37	60.3	39.8	2.8	
17 (a)	20	36.0	23.8	3.8	6.6 subcutaneously
(b)	28	72.0	47.5	19.5	
20 (a)	22	35.4	23.4	1.4	6.8 subcutaneously
(b)	29	68.7	45.3	16.3	
24 (a)	26	48.4	31.9	5.9	7.1 intravenously.
(b)	32	58.6	38.6	6.6	
27 (a)	11	14.4	9.5	1.5	4.5 intravenously
(b)	20	70.8	46.6	20.6	
29 (a)	18	19.7	13.0	5.0	3.8 subcutaneously
(b)	24	80.6	53.0	29.0	
30 (a)	19	39.1	25.8	6.8	6.4 intravenously
(b)	25	78.8	52.0	27.0	

(a) = before administration of sodium bicarbonate; (b) = after administration of sodium bicarbonate.

The influence of food on the hydrogen-ion concentration of the urine is shown by the work of Blatherwick,[9] and this influence is what might be expected from the base contents of the food. In a number

TABLE 5.

Hydrogen-Ion Concentration of Urine and Plasma Bicarbonate of Infants Fed on Diluted Cow's Milk.

Case No.	Hydrogen-Ion Concentration of Urine	Plasma Bicarbonate; C.c. of CO_2 Bound by 100 C.c. of Plasma at 0° and 760 Mm.	Case No.	Hydrogen-Ion Concentration of Urine	Plasma Bicarbonate; C.c. of CO_2 Bound by 100 C.c. of Plasma at 0° and 760 Mm.
1	5.7	54.3	9	4.8	57.4
2	6.2	58.6	10	6.8	72.0
3	5.8	58.7	11	5.7	60.3
4	5.7	50.9	12	4.8	56.1
5	5.4	63.0	13	5.5	59.3
6	6.0	60.0	14	6.9	79.8
7	6.3	67.0	15	5.2	63.7
8	4.9	58.0			
				Aver. 5.7	

TABLE 6.

Hydrogen-Ion Concentration of Urine Compared with Plasma Bicarbonate of Infants Fed on Protein Milk.

Case No.	Hydrogen-Ion Concentration of Urine	Plasma Bicarbonate; C.c. of CO_2 Bound by 100 C.c. of Plasma at 0° and 760 Mm.	Case No.	Hydrogen-Ion Concentration of Urine	Plasma Bicarbonate; C.c. of CO_2 Bound by 100 C.c. of Plasma at 0° and 760 Mm.
1	4.2	54.6	9	5.3	62.1
2	4.8	58.1	10	4.2	60.0
3	5.6	63.2	11	4.7	54.3
4	5.8	61.0	12	4.8	55.8
5	6.0	71.0	13	5.9	61.0
6	4.6	60.0	14	6.2	76.1
7	4.3	58.0	15	4.7	59.8
8	5.1	66.0			
				Aver. 5.08	

of our cases, the direct influence of food was shown by dietetic experiment. The results from two infants are shown in Table 8. In both

9. Blatherwick, N. R.: Arch. Int. Med. **14:** 409, 1914.

TABLE 7.

*Hydrogen-Ion Concentration of Urine Compared with Plasma Bicarbonate of Infants Fed on Dried Milk.**

Case No.	Hydrogen-Ion Concentration of Urine	Plasma Bicarbonate; C.c. of CO_2 Bound by 100 C.c. of Blood Plasma at 0° and 760 Mm.	Case No.	Hydrogen-Ion Concentration of Urine	Plasma Bicarbonate; C.c. of CO_2 Bound by 100 C.c. of Blood Plasma at 0° and 760 Mm.
1	4.1	53.6	9	4.7	54.0
2	4.6	55.0	10	4.7	57.0
3	4.7	56.7	11	5.9	69.4
4	5.0	55.3	12	4.6	58.1
5	5.0	58.6	13	4.8	56.2
6	4.8	59.0	14	5.4	66.4
7	5.3	66.0	15	5.7	62.0
8	5.0	63.0			
				Aver. 4.95	

Average total (Tables 5, 6 and 7), 5.24.

* The mixtures fed represented approximately 1.5 to 2 per cent. of fat, 4 to 5 per cent. of sugar and 3 to 5 per cent. of protein.

TABLE 8.

Influence of Food on the Hydrogen-Ion Concentration of the Urine.

Food	Hydrogen-Ion Concentration of Urine	Plasma Bicarbonate; C.c. of CO_2 Bound by 100 C.c. of Blood Plasma at 0° and 760 Mm.
Baby W.—		
Protein milk	4.8	57.6
Protein milk and potato gruel	6.3	63.7
Dried milk	4.3	56.7
Dried milk and potato gruel	6.0	59.3
Baby J.—		
Milk and water mixture	5.4	58.6
Milk and water mixture and potato gruel	6.3	61.4
Dried milk	4.6	58.4

instances, the addition of potato, which has a markedly alkaline ash, caused a reduction in the acidity of the urine. In both cases a similar, though less marked, influence on the alkaline reserve was observed.

Cases of Acidosis.—The results from fourteen cases of acidosis are shown in Table 9. The hydrogen-ion concentration of the urine is uniformly high, but the individual figures are no higher than some observed in infants without acidosis. With the correction of acidosis the hydrogen-ion concentration is lowered, as would be expected.

The results shown in Table 9 demonstrate the disadvantage of gaging the administration of sodium bicarbonate by the reaction of the urine. The use of such an indicator as litmus, which turns blue at a hydrogen-ion concentration of about 7, would necessitate raising the plasma bicarbonate much above normal. This has been shown by Palmer and Van Slyke.[10] Owing to this disadvantage, Marriott[11] suggests the use of cresol purple (dibrom-cresol-sulphone-phthalein) which changes color at about 5.3. This is much more satisfactory for this purpose and obviates increasing the plasma bicarbonate much above normal. Even this indicator, however, is not entirely satisfactory, for the urine may be too acid to change cresol purple despite the presence of a normal alkaline reserve. In Cases 1, 2, 8, 10, 12 and 14 (Table 9) the plasma bicarbonate was practically normal, and the urine was too acid definitely to change cresol purple. The same criticism, however, applies to estimation of the hydrogen-ion concentration of the urine.

On the other hand, the use of cresol purple is of definite negative value in excluding the presence or persistence of acidosis. If the urine causes the development of a magenta or purple color, acidosis can definitely be excluded.

The disadvantage in using an indicator to show when sufficient bicarbonate has been given is to a great degree hypothetical. Their use in determining the hydrogen-ion concentration of the urine could lead to no more than a slight increase of the plasma bicarbonate. While on general principles it would seem desirable to bring the plasma bicarbonate to normal and no more, yet it has not been demonstrated

10. Palmer, W. W., and Van Slyke, D. D.: J. Biol. Chem. **32:** 499, 1917.
11. Personal communication.

that a moderate increase in the plasma bicarbonate is necessarily harmful. In this connection Palmer cites a case observed by Tileston in which too much sodium bicarbonate was given and tetany resulted. In several of our cases the administration of large amounts of sodium bicarbonate was followed by tetany, or edema, or both. Whether

TABLE 9.

Comparison of Hydrogen-Ion Concentration of Urine with Plasma Bicarbonate in Cases of Acidosis.

Case No.	Hydrogen-Ion Concentration of the Urine	Plasma Bicarbonate; Volume per Cent. of CO_2 Bound by 100 C.c. of Plasma at 0° and 760 Mm.	Case No.	Hydrogen-Ion Concentration of the Urine	Plasma Bicarbonate; Volume per Cent. of CO_2 Bound by 100 C.c. of Plasma at 0° and 760 Mm.
1	4.6	36.3	8	4.1	37.0
	5.2	56.1*		4.8	60.0*
2	4.8	41.3	9	5.1	29.0
	5.1	60.0*		5.8	62.0*
3	4.3	30.0	10	4.2	33.8
	6.4	78.7*		5.1	58.6*
4	5.0	39.3	11	4.7	39.0
	7.0	94.0*		5.7	60.0*
5	4.1	32.8	12	4.2	28.7
	6.7	86.0*		5.2	58.8*
6	4.8	29.8	13	4.5	31.6
	5.4	54.3*		6.3	79.0*
7	5.0	40 0	14	4.7	39.3
	6.5	87.3*		5.1	54.8*

* After administration of sodium bicarbonate.

these conditions were a direct result of increasing the plasma bicarbonate above normal is difficult to determine. We have observed a number of cases in which sufficient bicarbonate was given to raise the plasma bicarbonate above normal but without the appearance of edema or tetany.

Despite the fact that there is no convincing evidence that an excess

of plasma bicarbonate is necessarily harmful, it would seem more nearly ideal to avoid raising the alkaline reserve much above normal. This can be done best by using the plasma bicarbonate as a therapeutic guide. If the determination of the plasma bicarbonate is not possible, the use of such an indicator as cresol purple is desirable. Even if with its use the alkaline reserve is raised somewhat above normal, this condition is of infinitely less danger than that of acidosis.

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A STUDY OF ATYPICAL TYPE II PNEUMOCOCCI.

BY ERNEST G. STILLMAN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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Study of the serological relation of strains of pneumococcus has shown that this organism may be classified by means of biological reactions into distinct types. From the first studies made it was evident that there existed three types of pneumococcus, the individual members of each type being alike in their immune reactions, the types themselves, however, differing sharply from one another. These types are now commonly known as Types I, II, and III. In addition to these fixed types there remain an indeterminate number of unrelated strains which for convenience have been grouped together and constitute the heterogeneous Type IV. More minute study of the last group indicates that it in turn can be further separated into a number of closely related immunological types.

The classification of pneumococcus was originally based upon immunological differences brought out by the reaction of protection. It later developed that the reaction of agglutination was as specific as the more time-consuming protection test, and recognition of this fact has led to its adoption as a routine method. In the course of the study of a large number of strains isolated from lobar pneumonia, it was observed that certain of these agglutinated atypically in Type II serum. Study of this phenomenon revealed the fact that these atypical Type II organisms possess partial antigenic characters common to the Type II pneumococcus, but vary from the typical representatives of this type by a diversity of relations among themselves, and by a lack of the reversibility of their immune reactions with the type organism. Because of these variations these organisms have been classified by Avery[1] as subvarieties of Pneumococcus Type II. In a study of ten such strains it was found possible to classify them

[1] Avery, O. T., *J. Exp. Med.*, 1915, xxii, 804.

into three distinct subtypes, IIa, IIb, and IIx. By means of agglutination, absorption, and protection experiments the members of Subtype IIa and IIb were found to possess immunity reactions identical with all other strains of the homologous types. Subtype IIx, however, consisted of a heterogeneous series of independent strains which showed neither cross-agglutination nor cross-protection.

The study reported in this paper was undertaken in order to determine the occurrence and frequency of atypical Type II pneumococci and their relation to lobar pneumonia, and to elaborate further their classification on the basis of their specific relations.

204 strains of pneumococci, all of which showed atypical agglutination in Type II serum, were studied. 77 strains were obtained from lobar pneumonia, 5 from postoperative pneumonia, 1 from meningitis, 5 from guinea pig pneumonia, 100 from normal mouths, 6 from convalescent Type I pneumonia, 3 from convalescent Type II pneumonia, 1 from convalescent Type III pneumonia, and 6 from dust.

In classifying these strains, agglutination and absorption reactions have been employed. In the agglutination reactions equal parts of broth cultures and immune sera were used, except where dilutions of sera were employed, when 0.1 cc. of culture in 0.9 cc. of immune serum diluted in normal salt solution was used. The agglutinations were incubated 2 hours in a water bath at 37°C., kept in the ice box over night, and readings made the next morning.

On the basis of specific agglutination in monovalent rabbit sera, the 204 strains were classified into twelve distinct groups as shown in Table I. From Table I it is seen that Subgroup IIb is the largest, representing 30 per cent of all strains studied. Next in order of frequency come Subgroups IIa, IIc, and IIm. These four groups comprise 73 per cent of all the strains classified. Subgroups IIa and IIb correspond to Avery's IIa and IIb, while the three strains originally placed in the IIx group were in the study of this series finally classifiable into Subgroups IIj, IIg, and IIe.

Only two strains showed cross-agglutination in the immune sera of a heterologous group. Strain St. 21 agglutinated promptly in serum of Subgroup IIm and slowly in that of Subgroup IIk. This culture was agglutinated by Serum IIm in a dilution of 1:160, but

only in a dilution of 1:80 by Serum IIk. A rabbit was immunized to Strain St. 21, and the type cultures, Nos. IIm and IIk, were tested for agglutinability in this serum.

From Table II it is seen that Culture IIm is agglutinated by Serum St. 21 in dilution of 1:300, while Culture IIk is affected by this serum only in a dilution of 1:100. Serum St. 21 was then exhausted

TABLE I.

Classification of 204 Strains of Type II Atypical Pneumococci.

Type.	Incidence.	
		per cent
IIa	38	19
IIb	62	30
IIc	28	14
IId	11	5
IIe	4	2
IIf	12	6
IIg	3	1
IIh	7	3
IIj	3	1
IIk	5	2
IIl	11	5
IIm	20	10

TABLE II.

Agglutination of Cultures IIk and IIm in St. 21 Serum.

Culture No.	Dilution of Serum St. 21.									
	1:10	1:20	1:40	1:80	1:100	1:160	1:200	1:240	1:300	Control.
IIk	++	++	++	+	+	–	–	–	–	–
IIm	++	++	++	++	++	++	++	++	++	–
St. 21	++	++	++	++	++	++	++	++	++	–

of agglutinins by absorption with Culture IIk, and its agglutinin titer determined for Cultures St. 21 and IIm.

From Table III it is seen that absorption of St. 21 sera with Culture IIk does not materially affect the agglutinins for Cultures St. 21 and IIm. Another lot of St. 21 sera was absorbed with Culture IIm. The results are shown in Table IV. From Table IV it is seen that

absorption of St. 21 serum with Culture IIm removes the agglutinins for both Strains St. 21 and IIk.

Strain P 65, which also showed cross-agglutination in heterologous sera, reacted slowly in Serum IIc and promptly in Serum IIm. This strain was tested like No. St. 21 by the absorption method and likewise showed that the reaction in Serum IIc was attributable to a minor agglutinin.

TABLE III.

Agglutination of Cultures St. 21 and IIm in St. 21 Serum Exhausted of Agglutinins for Strain IIk.

Culture No.	Dilution of Serum St. 21.									
	1 : 10	1 : 20	1 : 40	1 : 80	1 : 100	1 : 160	1 : 200	1 : 240	1 : 300	Control.
IIk	–	–	–	–	–	–	–	–	–	–
IIm	++	++	++	++	++	++	++	++	+	–
St. 21	++	++	++	++	++	++	++	+	+	–

TABLE IV.

Agglutination of Cultures St. 21 and IIk in St. 21 Serum Exhausted of Agglutinins for Strain IIm.

Culture No.	Dilution of Serum St. 21.									
	1 : 10	1 : 20	1 : 40	1 : 80	1 : 100	1 : 160	1 : 200	1 : 240	1 : 300	Control.
IIk	–	–	–	–	–	–	–	–	–	–
IIm	–	–	–	–	–	–	–	–	–	–
St. 21	–	–	–	–	–	–	–	–	–	–

Table V shows the source and type of the 204 strains studied. 77, or 38 per cent, were from cases of lobar pneumonia, 5 from postoperative pneumonia, and 1 from meningitis. 4 of the 5 strains from epidemic pneumonia in guinea pigs were isolated from guinea pigs in one city, while the 5th strain was from an epidemic in a different locality. All the guinea pig strains belonged to the same group. It has been shown that during convalescence from pneumonia the disease-producing types of pneumococci tend to disappear and are often replaced by Type IV or atypical Type II pneumococci, which have been previously shown to be the types most commonly found in

normal mouths. During convalescence the saliva of 6 Type I, 3 Type II, and 1 Type III patients showed atypical Type II pneumococci. If these are included, 110, or more than one-half of all the strains studied, may be considered as inhabitants of the normal mouth. 6 strains were recovered from dust.

From an examination of this table it is seen that the group may be divided according to the frequency of occurrence into two classes: (1) those found predominantly in disease; (2) those which occur more frequently in normal mouths.

TABLE V.

Source and Type of Atypical Type II Pneumococci.

Source.	Type.													
	a	b	c	d	e	f	g	h	j	k	l	m	Total.	
														per cent
Lobar pneumonia	33	12	2	5	1	2	1	7	2	2	6	4	77	38
Postoperative pneumonia	1	2			1							1	5	2
Meningitis												1	1	0.5
Guinea pig pneumonia			5										5	2
Normal mouths	2	38	18	6	2	10	2		1	2	5	14	100	49
Convalescent Type I	2	1	2							1			6	3
" " II		3											3	1
" " III			1										1	0.5
Total normal mouths	4	42	21	6	2	10	2	0	1	3	5	14	110	53
Dust		6											6	3
Total	38	62	28	11	4	12	3	7	3	5	11	20	204	

In the disease-producing class of atypical Type II organisms belong Subgroup IIa, with 33 strains out of a total of 38 from pneumonia, and Subgroup IIh, all of whose 7 strains are from pneumonia. Organisms of Subgroups IIb, IIc, IIf, and IIm are found frequently in healthy mouths, although they may also be found in association with disease. In some instances the groups comprise so few strains that it is impossible to determine their relation in either of these two classes.

During the last 3 years pneumococci have been isolated in 458 instances from 526 cases of lobar pneumonia admitted to the wards of

the Hospital of The Rockefeller Institute. These organisms have been typed according to the biological classification described by Dochez and Gillespie.[2] Of the 458 strains[3] obtained from disease, 52, or 11 per cent, were found to be atypical Type II organisms. Clough[4] in a study of 121 strains of pneumococci isolated from disease found 22, or 18 per cent. atypical Type II organisms. Sydenstricker and Sutton[5] in a study of 150 healthy individuals recovered atypical Type II pneumococci in 5 instances, and in 62 cases of pneumonia found this organism present in 12. They report a mortality

TABLE VI.

Type Incidence and Mortality of 59 Atypical Type II Pneumonias.

Type.	No. of cases of pneumonia.	Incidence.	No. of deaths.	Mortality.
		per cent		*per cent*
IIa	23	38	8	35
IIb	10	17	1	10
IIc	1	1	0	0
IId	5	8	3	60
IIe	1	1	0	0
IIf	1	1	0	0
IIg	0	0	0	0
IIh	7	12	4	57
IIj	2	3	0	0
IIk	1	1	0	0
IIl	5	8	1	20
IIm	3	5	1	33
Total..........	59		18	

of 31 per cent in pneumonia associated with atypical Type II pneumococci.

Table VI gives the mortality of 59 cases of lobar pneumonia from which atypical Type II pneumococci were isolated. 45 of these cases were in the Hospital of The Rockefeller Institute, 12 in the Presby-

[2] Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.

[3] Stillman, E. G., *J. Exp. Med.*, 1917, xxvi, 519.

[4] Clough, M. C., *Bull. Johns Hopkins Hosp.*, 1917, xxviii, 306.

[5] Sydenstricker, V. P. W., and Sutton, A. C., *Bull. Johns Hopkins Hosp.*, 1917, xxviii, 312.

terian Hospital, and 2 were not hospital cases. Although these figures are too small to warrant any conclusion, the total mortality of 32 per cent indicates a high pathogenicity and virulence. The three subgroups, IIa, IId, and IIh, showed the highest mortality.

DISCUSSION.

Avery, working with only ten strains, was able to differentiate two definite subgroups, IIa and IIb. In the present study of 204 strains, ten other groups have been recognized. It is possible that if a larger series were studied, still other groups would be found. It is significant, however, that all of the 204 strains were classified in one or another of these twelve groups. Only two strains showed cross-agglutination in the immune sera of a heterologous group and these reactions were shown to be due to the presence of minor agglutinins for one group. Indeed it is surprising that the specificity of these groups is as definite as it is.

Although atypical Type II pneumococci are found associated with about 11 per cent of cases of lobar pneumonia, they have an incidence of 18.3 per cent in normal mouths. But as certain subgroups, IIa and IIh, are encountered more frequently in relation to disease, it is probable that they have a greater pathogenicity. These same groups occur rarely in normal mouths. Just the opposite is the case with Subgroups IIb, IIc, IIf, and IIm, which are much more frequently encountered living a saprophytic existence in normal mouths than in association with disease. Type I and II pneumococci cause a large percentage of pneumonia, but occur rarely in normal mouths. On the other hand, the Type III and IV organisms, although occasionally found in association with pneumonia, occur frequently in normal mouths. Similarly the atypical Type II pneumococci exhibit the same division into parasitic and saprophytic types as is already recognized in the major classification.

SUMMARY.

1. At least twelve subgroups of atypical Type II pneumococcus may be recognized by specific agglutination reactions. They have been designated Subgroups IIa, IIb, IIc, IId, IIe, IIf, IIg, IIh, IIj, IIk, IIl, and IIm.

2. These subgroups have an incidence of 11 per cent in lobar pneumonia, and of 18 per cent in normal mouths.

3. Certain groups, IIb, IIc, IIf, and IIm, occur in normal mouths.

4. Subgroups IIa and IIh are met with largely in connection with disease.

5. The mortality of acute lobar pneumonia due to these atypical Type II pneumococci is fairly high—32 per cent.

The author acknowledges his indebtedness to Miss Miriam P. Olmstead of the Presbyterian Hospital for 41 of the cultures of pneumococci used in this study.

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THE OCCURRENCE OF BACILLUS INFLUENZÆ IN THROATS AND SALIVA.

BY IDA W. PRITCHETT AND ERNEST G. STILLMAN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

PLATES 10 TO 13.

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In the present paper are reported the facts obtained during an investigation of the bacteriology of influenza which included (1) the use of oleate hemoglobin agar, (2) the incidence of *Bacillus influenzæ* in uncomplicated and complicated cases of influenza and the associated types of pneumococci, (3) the occurrence of *Bacillus influenzæ* in convalescents, and (4) the occurrence of *Bacillus influenzæ* in the mouth secretions or throats of normal people.

The medium used in this work was Avery's oleate hemoglobin agar.[1] Agar of an average hydrogen ion concentration of 7.2 was used. As the domestic sodium oleate which was tested from time to time gave less consistent results, Kahlbaum's sodium oleate was used. This medium is especially favorable to the growth of Gram-negative organisms in influenza work. On these plates pneumococci do not grow and streptococci rarely grow, although some staphylococci show a scant growth (Figs. 1, 2, and 6). After 36 to 48 hours incubation several types of *Bacillus influenzæ* colonies are encountered on this medium. The characteristic colony of *Bacillus influenzæ* was clear, round, slightly glistening, convex, and discrete. The size varied from pin-point colonies, easily confused with diphtheroids or *catarrhalis* colonies, to large round colonies of 1 to 3 mm. in diameter. The larger characteristic colonies had a distinct nucleus which was much darker than the rest of the colony (Figs. 3, 4, and 7). The colonies were very sticky in consistency and would streak across the agar and follow the loop in long threads. The medium beneath the colony appears discolored after a colony is fished.

[1] Avery, O. T., *J. Am. Med. Assn.*, 1918, lxxi, 2050.

The colonies most readily confused on inspection with those of *Bacillus influenzæ* were those of *Micrococcus catarrhalis*, meningococcus, diphtheroids, and especially an unidentified organism called "Bacillus X." This organism was encountered in about two dozen individuals. The colony varied between 1 and 2 mm. in diameter, was clear, convex, and mucoid. The nucleus so common in the influenza colony was absent, but the colony left a slight discoloration on the agar. This bacillus is Gram-negative, takes the counterstain deeply, and when taken from agar surface cultures appears as long tangled threads, somewhat similar to the threads often seen in pure cultures of *Bacillus influenzæ*. In blood broth cultures it appears as a small fat bacillus without chain formation. It does not grow on plain sheep serum, or glucose agar. On blood agar the abundant growth appears as a clear, somewhat granular, fairly heavy film, with marked hemolysis of the red cells. This hemolysis is as marked as that produced by hemolytic streptococci (Fig. 5). Complete hemolysis is produced in blood broth after 24 hours incubation. After several days both blood broth and blood agar cultures turned brown and then blackish from the formation of methemoglobin. After 7 days incubation, litmus milk, to which a little blood had been added, showed an alkaline reaction with peptonization. No pathogenicity could be demonstrated for mice, rats, or rabbits.[2]

In all the cultures from convalescents and normal persons, and in many from the hospital patients, the organism was isolated in pure culture and was demonstrated to possess the typical morphology and shown to be strictly hemoglobinophilic. All evidence based simply

[2] The other organisms commonly found in cultures from the throat on this medium may be briefly described as follows in the frequency of their occurrence. (1) Gram-negative cocci of the *Micrococcus catarrhalis* group. The colonies are round, sharply defined, with a ridged or lined surface. Some colonies are so scaly that they lift off the medium as a pellicle; other colonies of this group will slide across the surface of the agar without breaking. (2) Staphylococci. Minute, pin-point colonies. (3) Diphtheroids. Minute, pin-point colonies. (4) Meningococci. The colonies are clear, round, and discrete. (5) Friedländer's bacillus. The colonies are white, mucoid, and occasionally confluent.

Occasionally in some plates a few Gram-positive streptococci may grow. These colonies are minute, dense, nucleated, and sharply defined. Pneumococci were never found and "spreaders" rarely grow.

on the presence of slender Gram-negative bacilli in films was discarded as of no value. The wide variations in the morphology of *Bacillus influenzæ* noted by Wollstein[3] were observed in this series. This study was made during the widespread epidemic of influenza which occurred in New York. The epidemic began about the middle of September, reached its height about the middle of October, and then gradually fell, though cases are still present in the city.

In Table I are shown the incidence of *Bacillus influenzæ* and the type of pneumococcus recovered from the patients admitted to the Hospital of The Rockefeller Institute, suffering from influenza, bronchopneumonia, and lobar pneumonia during the 14 weeks between September 16, and December 31, 1918. In these cases of influenza and pneumonia the presence of *Bacillus influenzæ* was determined in the majority of instances by blood agar plates and mouse inoculation. The selective medium, oleate hemoglobin agar, was used only in the latter part of the investigation.

TABLE I.

Incidence of B. influenzæ and Types of Pneumococci in Influenza and Pneumonia.

Disease.	No. of cases.	*B. influenzæ.*		Type pneumococcus.				No. of deaths.	Remarks.
				I	II	III	IV		
			per cent						
Uncomplicated influenza.	49	41	83+	0	2	2	17	0	
Influenza, with bronchopneumonia.	43	40	93	1	0	3	27	12	One case, Pneumococcus Types III and IV.
Bronchopneumonia.	6	6	100	0	0	1	4	0	
Lobar pneumonia.	20	11	55	3	2	6	7	7	One case, Friedländer's bacillus; one, staphylococcus.
Total..............	118	98	82	4	4	12	55	19	

From 49 cases of uncomplicated influenza *Bacillus influenzæ* was recovered in 41 instances, or 83 per cent. From the 43 cases which were complicated by bronchopneumonia this organism was cultivated

[3] Wollstein, M., *J. Exp. Med.*, 1915, xxii, 445.

in 40, or 93 per cent. The 6 cases of bronchopneumonia, which were probably late cases of influenza, all showed influenza bacilli. The incidence of *Bacillus influenzæ* is much lower in the 20 cases of lobar pneumonia, as only 11 cases, or 55 per cent, were positive.

The distribution of the types of pneumococci in these cases is especially interesting. Whereas normally pneumococci of Types I and II are found associated with over 60 per cent of the cases of lobar pneumonia, in the cases of bronchopneumonia complicating influenza they are rare in comparison with Type III and IV pneumococci, which are normally and commonly found in the mouths of healthy persons.

In order to determine how many normal persons and those convalescent from influenza harbor influenza bacilli, a study was made of the personnel of the Laboratories and the Hospital of The Rockefeller Institute and the War Demonstration Hospital. From each individual a throat culture was taken with a sterile swab, which was then rubbed across a corner of the oleate hemoglobin plate. A small amount of mouth saliva was also collected in a sterile dish from each case. A loopful of saliva was later placed on a side of the plate. Each plate was streaked with a loop according to the Sunburst method[4] (Figs. 8 and 9). The plates were examined for *Bacillus influenzæ* after an incubation period of from 36 to 48 hours at 37°C.

From Table II it is seen that of 54 convalescent cases, 25, or 46 per cent, harbored *Bacillus influenzæ*. The length of time that had elapsed between recovering from influenza and the date of the culture varied from 1 week to 4 months. The shortest period in which a convalescent had apparently ceased to be a carrier of *Bacillus influenzæ* was 1 week. The longest period during which a convalescent was known to carry *Bacillus influenzæ* was 3 months. The average carrying period, as fixed by the time of taking the cultures, was about 6 weeks. Of 177 persons who gave no history of having had influenza, 74, or 42 per cent, carried *Bacillus influenzæ*. The total incidence of influenza carriers among the 231 normals and late convalescent individuals was 99, or 43 per cent.

[4] Standard technique for meningococcus carrier detection; adopted by the Medical Department of the U. S. Army, U. S. Navy, and U. S. Public Health Service.

In the above cases both throat and saliva cultures were made on oleate plates.

In all the 231 cases here represented, 73 showed a positive culture of *Bacillus influenzæ* from a throat swab (Table III). In each instance only a single throat culture or sputum culture was taken. 55

TABLE II.

Incidence of B. influenzæ in Convalescents and in Normal Individuals.

Individuals.	No.	Positive cases.	
			per cent
Convalescents	54	25	46
Normals	177	74	42
Total	231	99	43

TABLE III.

Comparison of Throat and Saliva Cultures.

Individuals.	Throat +; saliva −.	Throat −; saliva +.	Throat +; saliva +.	Throat −; saliva −.	Total.
Convalescents	14	6	5	29	54
Normals	30	20	24	103	177
Total	44	26	29	132	231

TABLE IV.

Incidence of Positive Carriers among Vaccinated and Non-Vaccinated Normal Individuals.

Vaccinated.		Non-vaccinated.		Vaccinated.		Non-vaccinated	
Negative.	Positive.	Negative.	Positive.	Total.	Positive.	Total.	Positive.
					per cent		*per cent*
30	16	73	58	46	35	131	44

positive cultures of *Bacillus influenzæ* were obtained from saliva. This would seem to indicate that throat cultures are on the whole preferable to saliva cultures as a means of isolating *Bacillus influenzæ* from normal individuals or late convalescents. It must, however, be pointed out that in 26 instances *Bacillus influenzæ* was recovered from the saliva only, the throat cultures being negative.

Of the 177 normal people studied, 46 had received influenza vaccine. Of these, 35 per cent were positive carriers as compared with an incidence of 44 per cent among the 131 non-vaccinated individuals (Table IV). These observations were based on single cultures in each case. Thus it is apparent that there is no striking relation between vaccination and the carrier state. The importance of our observations is greatly lessened by the fact that many of those vaccinated had received their vaccine elsewhere and were as a rule unable to give any accurate information as to the dosage used.

DISCUSSION.

As a result of this study it is evident that during the influenzal epidemic period *Bacillus influenzæ* could be easily recovered from throats and saliva when a selective medium was used. The chief difficulty of isolating influenza bacilli from throats is analogous to that encountered in isolating meningococci from the nasopharynx; namely, that other organisms tend to overgrow *Bacillus influenzæ* just as they overgrow the delicate meningococci. The frequent occurrence of other Gram-negative bacilli, which morphologically may be easily confused with *Bacillus influenzæ*, renders the diagnosis of this organism from films alone valueless.

The high incidence of cultivation of *Bacillus influenzæ* from the upper respiratory tract of cases of influenza and bronchopneumonia during the epidemic is a point in favor of the view that this organism may be of significance in the disease in question. The fact that the incidence was high during this period in the cases of lobar pneumonia is not necessarily opposed to this view. Convalescent patients and normal individuals show about the same per cent of positive findings. It is noticeable that during the latter part of December, coincident with the ebb of the epidemic, the number of positive cultures decreased. The types of pneumococci found associated with influenza cases are of interest. Whereas normally 60 per cent of the cases of lobar pneumonia are associated with the presence of Type I and Type II pneumococci, these types are infrequently encountered in the pulmonary complications of influenza. On the contrary, the types which occur frequently in normal mouths, Types III and IV, are

usually recovered from the cases of bronchopneumonia which develop following influenza. At the same time a considerable number of the cases of lobar pneumonia occurring during this period were associated with the presence of pneumococci of Types I and II.

From this study it is clear that a diagnosis of *Bacillus influenzæ* is valueless if based only on the evidence obtained from direct films of saliva, throats, or cultures. It is also apparent that *Bacillus influenzæ* may be recovered in pure culture from a large percentage of acute cases of influenza, from convalescents, and from normal persons, if a suitable differential medium is used.

CONCLUSION.

1. Oleate hemoglobin agar is a good selective culture medium for *Bacillus influenzæ*.

2. *Bacillus influenzæ* has been cultivated from the mouths of 93 per cent of cases of influenza and bronchopneumonia.

3. *Bacillus influenzæ* was present at the time of this study in the mouths of 43 per cent of normal individuals.

4. The types of pneumococci found associated with the complicating bronchopneumonias of influenza are the types which are usually found in normal mouths.

EXPLANATION OF PLATES.

Plate 10.

Fig. 1. Plain blood agar plate. The lateral sectors show growth of pneumococcus and *Streptococcus hæmolyticus*. The lower sector shows *B. influenzæ*; growth is present, but not visible in the photograph.

Fig. 2. Oleate hemoglobin agar plate. Corresponding sectors are planted with the same organisms as in Fig. 1 and show enhanced growth of *B. influenzæ* in the lower sector and complete inhibition of growth of pneumococcus and *Streptococcus hæmolyticus*.

Plate 11

Fig. 3. Pure culture of *B. influenzæ* on oleate hemoglobin agar showing large nucleated colonies after 36 hours incubation.

Fig. 4. Throat culture from a case of lobar pneumonia on oleate hemoglobin agar showing large nucleated colonies of *B. influenzæ* after 36 hours incubation.

Fig. 5. Blood agar plate showing hemolysis produced by hemolytic streptococcus (top) and that produced by "Bacillus X" (bottom).

PLATE 12.

FIG. 6. Growth of *B. influenzæ* on oleate hemoglobin agar; 15 hours growth at 37°C.

FIG. 7. Throat culture from a normal person on oleate hemoglobin agar showing large colonies of *B. influenzæ*; 36 hours growth at 37°C.

PLATE 13.

FIG. 8. Throat culture from a case of lobar pneumonia on oleate hemoglobin agar showing large colonies of *B. influenzæ*; 36 hours incubation at 37°C.

FIG. 9. Throat culture from a normal person on oleate hemoglobin agar showing many small colonies of *B. influenzæ;* 36 hours incubation at 37°C.

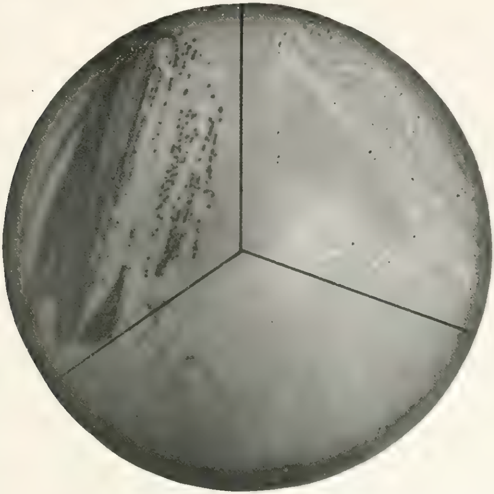


FIG. 1.

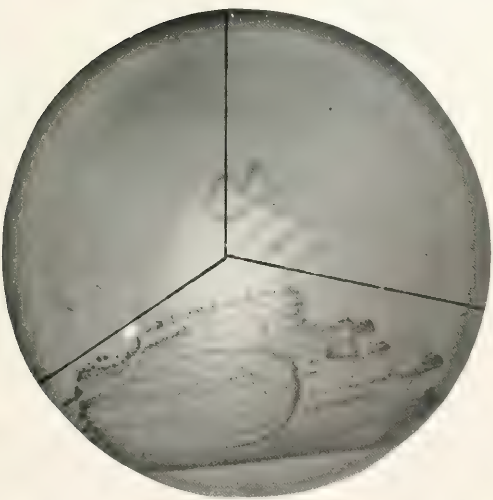


FIG. 2.

(Pritchett and Stillman: *B. influenzæ* in throats and saliva.)

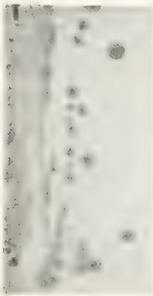


FIG. 3.

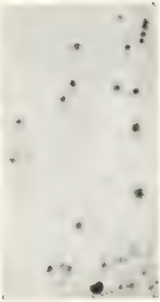


FIG. 4.

FIG. 5.

(Pritchett and Stillman: *B. influenzæ* in throats and saliva.)

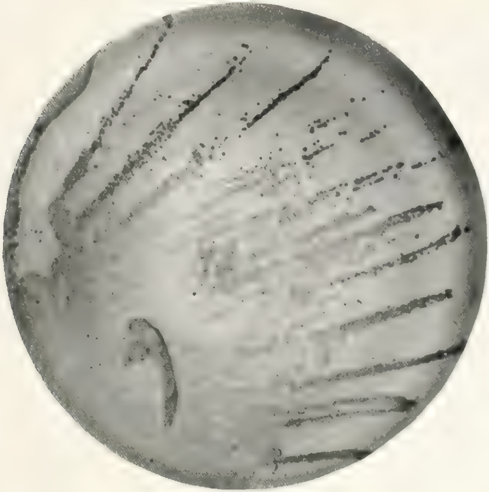


FIG. 6.

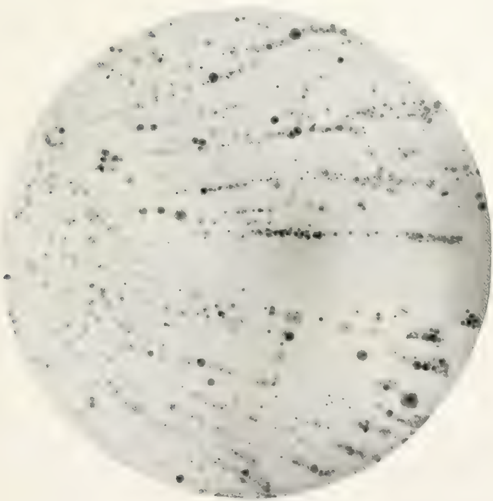


FIG. 7.

(Pritchett and Stillman: *B. influenzæ* in throats and saliva.)

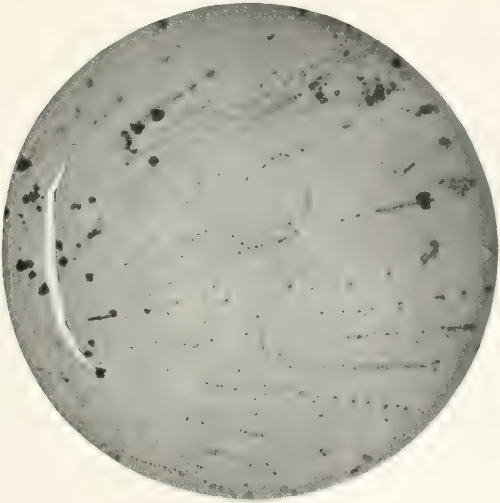


Fig. 8.

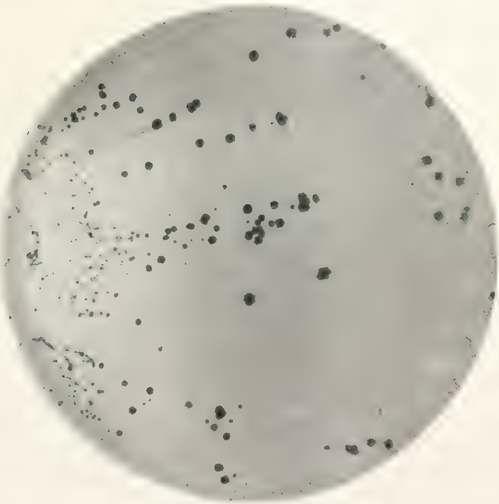


Fig. 9.

(Pritchett and Stillman: *B. influenzæ* in throats and saliva.)

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, April, 1919, Vol. xxxvii, No. 4, pp. 511–517.]

NOTE ON THE STABILIZATION OF DILUTE SODIUM HYPOCHLORITE SOLUTIONS (DAKIN'S SOLUTION).

BY GLENN E. CULLEN AND ROGER S. HUBBARD.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 22, 1919.)

The preparation of dilute sodium hypochlorite solutions electrolytically is so economical and convenient that it was desirable to determine a simple method of stabilizing it for use as Dakin's solution.

We have compared the influence of various substances on the rate of decomposition of dilute sodium hypochlorite solutions.

Dakin[1,2] set the upper limit of alkalinity of clinical hypochlorite solutions at the end-point of powdered phenolphthalein and used buffer salts to maintain the alkalinity below this limit. He neutralized with boric acid the excess alkalinity of the filtrate from the sodium carbonate and bleaching powder. The original Dakin's solution, therefore, contained both carbonate and borate buffers. Daufresne[3] modified Dakin's method by substituting sodium bicarbonate for the boric acid. The resulting solution contained only carbonate buffers.

Cullen and Austin,[4] studying the alkalinity of dilute sodium hypochlorite solutions containing carbonates, found that the end-point with powdered phenolphthalein was at a hydrogen ion concentration of about 10^{-10} N (pH 10), and that a reduction of the alkalinity below a pH of 9 made the solution too unstable to be of clinical use. The alkalinity of Dakin's solution, therefore, must

[1] Dakin, H. D., *Brit. Med. J.*, 1915, ii, 318.

[2] Dakin, H. D., and Dunham, E. K., Handbook of antiseptics, New York, 2nd edition, 1918, 116.

[3] Daufresne, M., *Presse méd.*, 1916, xxiv, 474.

[4] Cullen, G. E., and Austin, J. H., *J. Biol. Chem.*, 1918, xxxiv, 553.

be between 100 and 1,000 times that of water. The lower limit of alkalinity may be approximately detected by the use of alcoholic solution of phenolphthalein or *o*-cresolphthalein. The desirability of maintaining the alkalinity within this zone has been further emphasized by the observation that dilute hypochlorite solutions with an alkalinity less than indicated by a pH of 9, as well as those with an alkalinity greater than indicated by a pH of 10, are much more irritating than those with a pH between 9 and 10 (Cullen and Taylor).[5]

Daufresne has stabilized electrolytic sodium hypochlorite solutions by the use of small amounts of NaOH.[6] Although such solutions, unlike those originally described by Dakin, contain no buffer salts, they nevertheless conform to his requirements in concentration and alkalinity, and, therefore, it appears justified to call them Dakin's solutions.

EXPERIMENTAL.

The following substances were tested:

Borates.—Since Dakin used the highly efficient buffer action of borates, it was logical to test the stabilizing influence of borates on electrolytically prepared sodium hypochlorite solutions. Fortunately, the addition of borax ($Na_2B_4O_7.10H_2O$) so adjusted the reaction that there was no color to powdered phenolphthalein, but definite color with alcoholic phenolphthalein or *o*-cresolphthalein. Borax was added in a concentration of from 0.25 to 1.5 per cent.

Carbonates.—Three sets of carbonate and bicarbonate mixtures, having pH values of 10, 9.5, and 9 respectively, were prepared and added to the test solution in concentrations of 0.5, 1, and 1.5 per cent. McCoy's[7] formula was used in calculating the ratio of carbonate to bicarbonate.

[5] Cullen, G. E., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 681.

[6] Personal communication. Daufresne adds NaOH to the solution in the proportion of 0.2 gm. of NaOH to 5 gm. of NaOCl; *i.e.*, if he prepared a 1 per cent NaOCl solution he would add 0.5 gm. per liter. This solution keeps for months. He uses a special cell with platinum and silver electrodes and probably has much less secondary decomposition produced with this cell.

[7] McCoy, H. N., *Am. Chem. J.*, 1903, xxix, 437.

$$\frac{NaHCO_3}{Na_2CO_3} = C_H \times \frac{0.8}{6.0 \times 10^{-11}}$$

that is, for pH of 10.0 1 molecule Na_2CO_3 to 1.3 molecules $NaHCO_3$
" " 9.5 1 " " " 4.2 " "
" " 9.0 1 " " " 13.3 " "

Na_2CO_3 alone, of course, makes the solution too alkaline, and $NaHCO_3$ alone accelerates the rate of decomposition by greatly reducing the alkalinity.

In order to determine the pH of the hypochlorite solutions after the addition of carbonate, 10 cc. samples were decomposed with

TABLE I.

1 Per Cent NaOCl Made from 8 Per Cent NaCl Diluted to 0.5 Per Cent NaOCl with 2 Per Cent Carbonate Solution and Water.

pH of 2 per cent carbonate solution.	Carbonate. Final concentration.	Final pH of hypochlorite solution.
	per cent	
10	0.25	8.9
10	0.5	9.5
10	0.75	9.7
10	1.0	9.8
9.9	0.25	8.7
9.9	0.5	9.2
9.9	0.75	9.6
9.9	1.0	9.7
9.6	0.5	8.8
9.6	1.0	9.4

hydrogen peroxide and titrated with 0.1 N HCl to methyl orange and phenolphthalein. As shown in Table I the pH of the hypochlorite solutions after the addition of 1 per cent carbonate is about 0.2 pH less than calculated for the ratio of sodium carbonate and bicarbonate added.

With impure sodium chloride or with water of high calcium or magnesium content a rather heavy cloud will form in the solution after the addition of carbonate. This will settle out on standing or may be removed by filtration.

Sodium Hydroxide.—NaOH was added to the solution in amounts from 0.15 to 0.3 gm. per liter. Over 0.03 per cent makes the solution too alkaline; that is, it gives color with powdered phenolphthalein.

These substances were tested in two ways: First, current was passed through an electrolytic cell, filled with brine, just long enough to produce 0.5 per cent NaOCl. The solution was withdrawn from the cell, thoroughly mixed, and measured portions were placed in dark brown bottles containing the desired quantity of hydroxide or buffer. Second, the current was passed until the NaOCl was about 0.7 to 0.8 per cent, that is, near the crest of the curve.[8] The solution was then removed and diluted to 0.5 per cent with water. In this case the stabilizing substances were added in concentrated solutions. With this procedure more secondary products are formed (see following paper), the solutions are more unstable, and the test, therefore, more severe.

Several experiments were run under varying conditions. In some the solutions were kept in brown bottles; in others they were exposed to light to accelerate decomposition.

Two representative experiments are given in Tables II and III. Any change in the alkalinity of these solutions during the period of the experiments was within the range of alkalinity indicated by the end-points of alcoholic and powdered phenolphthalein and *o*-cresolphthalein.

It is evident from our experiments that 0.2 to 0.3 gm. per liter of sodium hydroxide, borax in concentration of 0.25 per cent up, carbonate mixture of pH 10 from 0.5 per cent up, and carbonate mixture of pH 9.5 from 1.0 per cent up, maintain dilute NaOCl solution within the requirements of Dakin's solution for at least a week. The pH 9 carbonate mixture allows somewhat more rapid decomposition. If sodium hydroxide is used, care must be taken that the amount is accurately measured, or the alkalinity may be made dangerously high. The use of borax combines a maximum of convenience and safety.

[8] Cullen, G. E., and Hubbard, R. S., *J. Biol. Chem.*, 1919, xxxvii, 519, Fig. 2, Curve C.

TABLE II.

10 Liters of 3 Per Cent NaCl. 110 Volts. 14 to 16 Amperes. Initial Temperature 18°C. 15 Minutes. NaOCl = 0.5 Per Cent.

Treatment of solution.		Sodium hypochlorite concentration.	
Substance.		Initial.	After 7 days in brown bottle.
	per cent		
Control		0.5	0.35
Borax	0.5	0.5	0.48
"	0.25	0.5	0.465
NaOH	0.3	0.5	0.49
"	0.2	0.5	0.46
"	0.015	0.5	0.42
pH 10 carbonate	1.0	0.5	0.49
" 9.5 "	1.0	0.5	0.45
" 9 "	1.0	0.5	0.41

TABLE III.

10 Liters of 3 Per Cent NaCl. 110 Volts. 20 Minutes. Initial Temperature 7°C 0.75 Per Cent NaOCl Diluted to 0.5 Per Cent.

Treatment of solution.		Sodium hypochlorite concentration.		
Substance.		Initial.	After 7 days in colorless bottle.	After 26 days in brown bottle.
	per cent			
Control		0.5	0.29	0.09
Borax	1.5	0.5	0.48	0.50
"	1.0	0.5	0.48	0.45
"	0.5	0.5	0.47	0.41
NaOH	0.03	0.5	0.49	0.48
"	0.02	0.5	0.48	
"	0.015	0.5	0.42	0.23
pH 10 carbonate	1.5	0.5		0.49
" 10 "	1.0	0.5	0.49	0.48
" 10 "	0.5	0.5		0.47
" 9.5 "	1.5	0.5		0.48
" 9.5 "	1.0	0.5	0.45	0.47
	0.5	0.5		0.43

DISCUSSION.

The solvent action of the sodium hypochlorite solution is an essential factor in its germicidal efficiency. The necrotic tissue, pus, etc., that protect the bacteria from many antiseptics are dissolved away by the hypochlorite solution. Fiessinger and his coworkers have attributed this solvent action mainly to the alkalinity of the solution, but Taylor and Austin[9] have shown that within the zone pH 9 to pH 10 the solvent action is primarily due to the hypochlorite. However, when no alkali was added to the hypochlorite solution and the alkalinity was due entirely to the dissociation of sodium hypochlorite, solvent action ceased when the hypochlorite concentration was reduced below 0.2 per cent.

Solution of the protein material probably takes place in two steps: first, the chlorination of the protein, and second, the formation of soluble sodium salts of the chlorinated proteins. The chlorination of the protein, as indicated by the rate of the decomposition of the hypochlorite solution, is rapid when large amounts of pus and necrotic tissue are present, and the initial 0.5 per cent concentration is maintained in the wound for only a few minutes. When the alkalinity is maintained by buffer salts, the solvent action occurs not only during this period but presumably until all the products capable of reacting are changed to the soluble sodium salts. When the alkalinity is due to small quantities of free alkali, as sodium hydroxide, the concentration of alkali should be sufficient to maintain the alkaline reaction long enough for efficient solvent action. On the other hand, the concentration must not be so great that free alkali remaining unneutralized in the wound is sufficient to cause irritation. The concentration used by Daufresne—0.2 to 0.3 gm. per liter—seems to satisfy these conditions. Experience at the War Demonstration Hospital of The Rockefeller Institute has not yielded clinical evidence to warrant choosing between the two types of hypochlorite solutions provided they conform to Dakin's conditions of hypochlorite concentration and alkalinity.

[9] Taylor, H. D., and Austin, J. H., *J. Exp. Med.*, 1918, xxvii, 155.

SUMMARY.

There are two methods of applying Dakin's principle of low alkalinity: first, by maintaining the alkalinity at a pH of 9 to 10 by means of buffer salts, such as carbonate or borates; second, by maintaining a similar alkalinity by means of *small* amounts of alkali. Both methods are efficient.

0.5 per cent sodium hypochlorite, prepared by the electrolysis of sodium chloride, may be conveniently stabilized for use as Dakin's solution by the addition of 0.5 per cent borax, of 0.5 to 1.0 per cent of carbonate mixtures of pH 10 to 9.5, or by the addition of 0.2 gm. of sodium hydroxide per liter (Daufresne).

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, April, 1919, Vol. xxxvii, No. 4, pp. 519-523.]

NOTE ON THE ELECTROLYTIC PREPARATION OF DILUTE SODIUM HYPOCHLORITE SOLUTIONS (DAKIN'S SOLUTION).

BY GLENN E. CULLEN AND ROGER S. HUBBARD.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, January 22, 1919.)

Although the electrolysis of brine is an old process, information in regard to it is scattered and hidden in the records and patent literature of industrial concerns. Dakin and Carlisle[1] have pointed out its convenience and economy in preparing dilute sodium hypochlorite solutions for disinfection purposes and have designed a simple cell. In instructing army surgeons in the various methods of preparing Dakin's solution, it was desirable for us to determine for ourselves the factors that were of practical importance in its electrolytic preparation. These results are presented for the convenience of other workers.

EXPERIMENTAL.

Choice of Cell.—It seemed desirable to use a cell that could be connected with ordinary 110 volt current, that did not require unusually heavy wiring or power, that was light and strong enough to be transportable with other military hospital equipment, and that was inexpensive. The cell described by Dakin and Carlisle answers these requirements, but we were spared the labor of making this cell by modifying cells already on the market to our purpose. These cells are entirely similar to that described by Dakin and Carlisle, except that the electrode area is smaller in proportion

[1] Dakin, H. D., and Carlisle, H. G., *J. Roy. Army Med. Corps*, 1916, xxvi, 209. See also Dakin, H. D., and Dunham, E. K., Handbook of antiseptics, New York, 2nd edition, 1918, 116.

to the volume of solution. The twenty-three intermediate electrodes contained 30 square inches of Acheson graphite. The cell[2] used in these experiments held 10 liters of brine and required between 20 and 35 amperes.

The results are presented as curves, with the omission of the tables from which they were derived.

Influence of Temperature.—In order to determine the effect of the initial temperature of the solution, the current through the cell was maintained constant at 20 amperes by an external resistance.[3] This

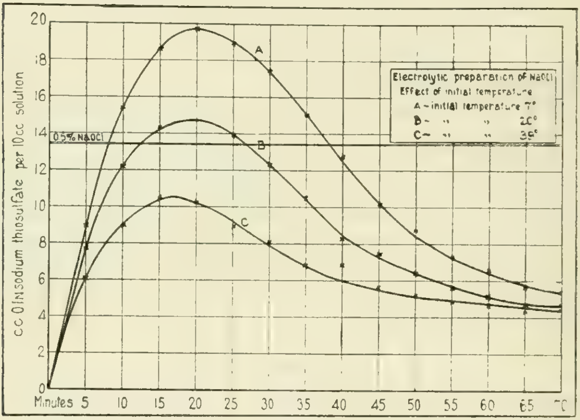


Fig. 1.

eliminates the influence of current fluctuations due to change in internal resistance. Fig. 1 shows the results with initial temperatures of 7, 20, and 39°.

Influence of Salt Concentration.—Increase in salt concentration will, of course, lessen the internal resistance of the cell and con-

[2] This cell was furnished by courtesy of the Electro Chemical Company of Dayton, Ohio.

[3] The resistance units used to control the lights in theaters are convenient for this work.

sequently increase the production of sodium hypochlorite per unit of time. The curves in Fig. 2 show the production of sodium hypochlorite with different salt concentrations under actual operating conditions, with no external resistance.

The 3 per cent solution approximates sea water, and 6 per cent is the strength recommended by the makers. More concentrated solutions may, of course, be used and with them higher concentration of sodium hypochlorite and increased current efficiency may be obtained.

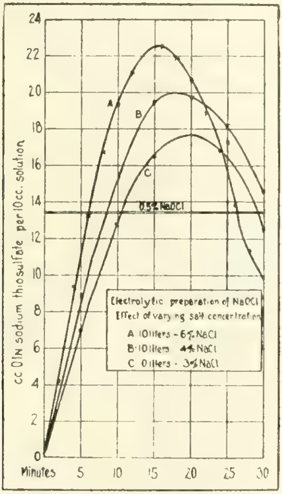
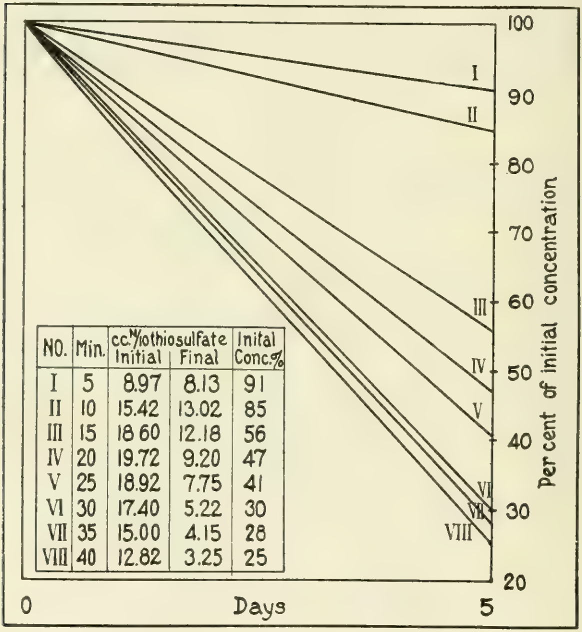


FIG. 2.

Rate of Decomposition of Electrolytic Sodium Hypochlorite.—In the experiments shown above samples of solution were removed from the cell at each of the points shown, titrated, and a portion was set aside for determination of stability. The solution became increasingly unstable with increase in hypochlorite concentration. The results obtained from Curve A, Fig. 1, run at initial temperature of 7°, are plotted on Fig. 3. This increased rate of de-

composition is due to increase in secondary products rather than to the temperature at time of sampling, for in one series in which the samples were all cooled to the same temperature the results were essentially similar.



NO.	Min.	cc. N/10 thiosulfate Initial	Final	Inital Conc. %
I	5	8.97	8.13	91
II	10	15.42	13.02	85
III	15	18.60	12.18	56
IV	20	19.72	9.20	47
V	25	18.92	7.75	41
VI	30	17.40	5.22	30
VII	35	15.00	4.15	28
VIII	40	12.82	3.25	25

Fig. 3.

DISCUSSION.

It would seem desirable to construct from experimental runs, curves similar to those of Figs. 1 and 2, for each cell. Then, from the initial temperature of the solutions, the time required for a solution of given concentration may easily be determined. Care

should be taken that the cell is not operated beyond the peak of the production curve. Ordinarily 3 per cent NaCl (or sea water) is satisfactory, but if necessary to operate with relatively warm solution the salt concentrations should be increased.

Since the solution, as it comes from the cell, decomposes quickly, it must be stabilized for use as Dakin's solution. As determined in the preceding paper, this can best be accomplished by adding either 0.5 per cent borax, or 0.5 to 1.0 per cent of a mixture of carbonate and bicarbonate of pH 10 to 9.5, or 0.02 per cent sodium hydroxide.

This solution must give no color with powdered phenolphthalein but should give a definite red flash with alcoholic phenolphthalein solution. It should be protected from light and should be titrated frequently.

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A SIMPLIFICATION OF THE McLEAN-VAN SLYKE METHOD FOR DETERMINATION OF PLASMA CHLORIDES.

BY DONALD D. VAN SLYKE AND JOHN J. DONLEAVY.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, February 19, 1919.)

The method as originally published[1] required for each determination three filtrations as a preliminary to the titration; one after coagulation of the proteins with heat and magnesium sulfate, a second after treatment with charcoal to remove the last traces of protein, and a third after precipitation of the Cl with standard silver nitrate.

We have found that the three filtrations can be condensed into one by addition of picric acid to the standard silver nitrate. The technique consequently involves only one filtration and the measurement of an aliquot part of the filtrate for titration.

Rappleye[2] has recently published a method for plasma chloride determination, based on the Volhard titration, involving a technique as simple as ours, and in fact practically identical, *viz.* a precipitation with silver nitrate and titration of the filtrate, the difference being that Rappleye titrates the excess silver with sulfocyanate instead of iodide. We have obtained entirely accurate results with Rappleye's method. The choice between it and the iodide titration, therefore, depends upon individual preference.

Solutions.—The standard silver nitrate solution (Solution I, p. 438 of the original paper)[3] is made up to contain, in addition to the

[1] McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxi, 361.

[2] Rappleye, W. C., *J. Biol. Chem.*, 1918, xxxv, 509.

[3] McLean, F. C., and Van Slyke, D. D., *Studies from The Rockefeller Institute for Medical Research*, 1916, xxiii, 436.

silver nitrate and nitric acid, 7.5 gm. of picric acid per liter. This solution precipitates both proteins and chlorides simultaneously.[4]

The composition of the indicator solution, containing nitrite, citrate, and starch, is unchanged from that described in the original paper. It may be well to emphasize that when the starch is dissolved the solution must be not merely heated, but *boiled* for several minutes, or it will not give a satisfactory end-point. If starch other than the "soluble" variety is used the boiling should continue for an hour.

The $\frac{M}{58.5}$ KI solution, of which 1 cc. is equivalent to 1 mg. of NaCl, has been replaced by $\frac{M}{73.1}$ KI solution, of which 1 cc. is equivalent to 0.8 mg. of NaCl. The greater dilution of the KI solution is conducive to slightly increased accuracy in the titration; and the fact that it is equivalent to 0.8 mg. of NaCl per cc. simplifies the calculation to a single subtraction (see below). The KI solution is standardized to make 2.5 cc. equivalent to 1 cc. of the $AgNO_3$ solution.

Standardization of the KI Solution.—The solution contains 2.27 gm. of pure KI per liter. It is made up to contain 2.4 gm., and diluted to the extent indicated by a preliminary titration. 5 cc. of the silver nitrate solution, measured with a pipette which has an error not greater than 0.01 cc. (delivers 4.97 gm. of water at 20°), are mixed with 5 cc. of the starch-citrate solution and 5 cc. of water, and iodide is run in from a burette to a permanent blue end-point. The amount required should be 12.65 cc., 12.50 cc. being required to precipitate the standard silver solution, and 0.15 cc. additional to give the end-point (see below under "End-Point").

[4] The silver nitrate solution contains per liter 5.812 gm. of pure fused $AgNO_3$, 250 cc. of HNO_3 (specific gravity 1.42), and 7.5 gm. of picric acid.

The starch-citrate nitrite solution contains per liter 446 gm. of crystalline sodium citrate ($Na_3C_6H_5O_7$) + $5\frac{1}{2}$ H_2O, 20 gm. of $NaNO_2$, and 2.5 gm. of soluble starch. The starch is first dissolved in about 500 cc. of boiling water, to which the citrate and nitrite are then added, the solution being finally made up to 1 liter. 4 cc. of this solution contain sufficient citrate to react with the acid in 1 cc. of HNO_3 of 1.42 specific gravity, the resulting solution having the optimum acidity for production of the blue starch-iodine end-point.

Determination of Chlorides in Plasma.—2 cc. of oxalate or citrate plasma are drawn into a dry pipette calibrated to *contain* 2 ± 0.005 cc. (The pipette must weigh 1.994 ± 0.005 gm. more when filled with water at 20° than when empty and dry.) The plasma is run into a 50 cc. measuring flask half full of water, and the pipette is rinsed by drawing the water up into it twice. 10 cc. of the standard silver nitrate-picric acid solution are added, and the mixture is diluted to the 50 cc. mark and shaken at intervals for several minutes, until coagulation is completed. The addition of a drop or two of caprylic alcohol prevents foaming and facilitates coagulation. The solution is passed through a dry, chloride-free filter, the first portion of filtrate being passed through, if necessary, a second time to remove turbidity completely. 20 cc. duplicate portions of the filtrate are measured with a calibrated pipette into 100 cc. Erlenmeyer flasks, 4 cc. of the starch-citrate indicator solution are added to each, and the standard KI is run in from a burette until a permanent blue end-point is obtained.

If it is desirable to use less than 2 cc. of plasma, 1 cc. measured within ± 0.002 cc. may be precipitated with 5 cc. of standard silver solution and diluted to 25 instead of 50 cc., the filtrate yielding only one 20 cc. portion instead of duplicates.

The End-Point.—Only a *permanent* and unmistakable blue is taken as the end-point. If the iodide is run in rapidly near the end of the titration, iodine may be formed more rapidly than the silver nitrate precipitates it, and a false end-point reached which disappears after a few seconds shaking. If, towards the end of the titration, the iodide is added in the usual manner, slowly, the blue shade caused by each drop disappears as soon as the solution is rotated, until the genuine end-point is reached. The latter is permanent, and in fact deepens with time.

The first appearance of the end-point is observed easily against a white background, but even more readily against one which matches the light yellow color of the silver iodide precipitate and the picric acid-tinged solution. We usually perform the titration over a sheet of light yellow paper which lies on the table beneath the burette.

The fact that 0.15 cc. of excess KI solution is required to give the end-point does not detract from the accuracy of the titration, because, with a given volume of solution, the required excess is constant and sharply defined. The end-point is not approached gradually, but appears suddenly on the addition of the last drop of the 0.15 cc. excess of KI. This may be demonstrated on a control solution by mixing 1 cc. of HNO_3 of 1.42 specific gravity with 4 cc. of starch-citrate indicator, diluting up to 25 cc., and titrating with the KI solution. The first 0.10 cc. produces no color at all, but the next drop suddenly causes a definite blue which deepens rapidly for several seconds. The amount of excess KI required to produce the end-point varies directly as the volume of the solution; consequently it is desirable to keep the volume at the end of the titration within approximately the same limits (25 to 30 cc.) in standardizing as in performing the analyses.

Calculation.—The calculation is very simple when standard solutions of the above concentration are used. The 20 cc. of filtrate used for titration represent 0.8 cc. of plasma, and the unprecipitated portion of an amount of $AgNO_3$ equivalent to 8 mg. of NaCl, or 10 mg. per cc. of plasma. Each cc. of KI used in the titration is equivalent to 1 mg. of NaCl per cc. of plasma. Hence the calculation simplifies to:

$$\left.\begin{array}{l}\text{Mg. NaCl per cc. plasma, or}\\ \text{Gm. " " liter "}\end{array}\right\} = 10.15 - \text{cc. KI}$$

The use of 10.15 instead of 10 cc. is due to the fact that 0.15 cc. excess of KI solution is required to give the end-point. If the 0.15 cc. of excess iodide required to give the end-point were neglected in the calculation, the error would be partially eliminated by neglecting it also in the standardization. It is as simple, however, to allow for it in the calculation and thereby eliminate it entirely.

Necessity for Calibrated Apparatus.—As compared with most determinations used in physiological and clinical work, exceptional accuracy is required in the estimation of plasma chlorides, for the reason that the significant figure is not so much the total amount as the relatively small difference between that and the normal

"chloride threshold" level of 5.62 gm. of NaCl per liter.[5] Consequently errors of more than 0.10 gm. per liter are undesirable. In order to prevent such errors it is necessary to check the accuracy of all pipettes, burettes, and measuring flasks used by calibration. Glass measuring apparatus, because of slow shrinkage after blowing or error in original calibration, is not, as obtained from the dealers, sufficiently accurate to be used for this determination without being checked.

Comparison of Results with Those of Original Method.—The following parallel determinations on a series of human plasmas by the original McLean-Van Slyke method and the present modification indicate that the results are identical.

Plasma No.	NaCl per liter.	
	McLean-Van Slyke method.	Present modification.
	gm.	*gm.*
1	5.90	5.90
	5.90	5.90
2	6.00	6.05
	6.04	6.02
3	6.00	6.00
	6.00	6.02
4	5.35	5.43
	5.35	5.38
5	5.35	5.40
	5.42	5.40
6	5.55	5.55
	5.50	5.52
7	5.68	5.65
	5.63	5.54
8	6.01	6.01
	6.01	6.03

[5] McLean, F. C., *J. Exp. Med.*, 1915, xxii, 212, 366.

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THE ACTION OF STROPHANTHIN ON THE LIVING CAT'S HEART.*

BY SAMUEL A. LEVINE, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

PLATES 23 TO 25.

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INTRODUCTION AND HISTORICAL REVIEW.

A review of the pharmacological literature concerning the action of the digitalis bodies reveals a complicated problem and conflicting views. Many difficulties in the way of clear understanding have resulted from the different conditions under which similar phases of this question were investigated, from the impossibility of translating the results of a study of one species to another, and from attempting to transfer the results of the study of one of the digitalis glucosides into terms of another. A prime difficulty consists in the fact that the fate of these drugs within the body is not understood, in respect either to their internal catabolism or to their excretion. This is true partly because much of the experimental work has been done on isolated frogs' hearts perfused in various ways. The interpretation of results from such experiments must be made carefully because they provide no opportunity for essential studies, such as excretion by the kidneys, combination or destruction of the drugs by other tissues, or for observing the influence of the extracardiac nervous control. For these reasons additional knowledge that will help to clarify any part of this problem is of importance, especially if it improves the technique of the clinical administration of digitalis.

Importance of the Concentration of Digitalis and of the Total Amount.—A review of the literature of this subject shows that one of the important problems relating to the digitalis glucosides concerns their mode of action. Does this depend on a physical or a chemical process? Many experiments on isolated frogs' hearts have given evidence that standstill of the ventricle is obtained in the same length of time even when the amount of the perfusion fluid is altered if the concentration of the active principle is kept constant. If the total amount of perfusion fluid is constant, however, the time for standstill will be inversely proportional to the concentration. Straub (1) and later Holste (2), using pure

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strophanthin, came to the conclusion that the action depended on the concentration to which the heart was exposed and not on the total amount of the drug. Grünwald (3), using digitalin and isolated frogs' hearts, found that although the concentration of the drug was the most important factor, the total amount of the solution and therefore of the drug, was of considerable influence in experiments where low dilutions were used. Straub (4) ascribed the discrepancy in Grünwald's results to the fact that while the latter used digitalin, he used pure strophanthin. Weizsäcker (5) agreed with Straub that although the amount of fluid was quite important in low dilutions of digitalin, the amount had no effect on the action of strophanthin. The lowest dilution of strophanthin he used, however, was 0.00025 per cent. Clark (6) and Issekutz (7) found marked differences with digitoxin and only slight, but definite ones with strophanthin when equal concentrations but different amounts were compared. The importance of the problem as to whether digitalis acts because of a certain concentration or because of a certain total amount is appreciated when one realizes that clinically the drug is given, neither to produce standstill of the heart, nor even to produce toxic effects, but rather in amounts so small as to be comparable with those low dilutions in which Grünwald, Clark, and Issekutz found that the total amount of the drug played some part. It is reasonable to suppose that when the drugs are used clinically in such small amounts and in such low dilutions the small amount taken up by the heart and perhaps by other tissues of the body would be sufficient definitely to alter the remaining concentration in the blood stream.

Influence of Temperature and Heart Rate.—There are many factors that influence the effects of the digitalis bodies on the heart. The effect of temperature on the rapidity of digitalis action has been thoroughly studied. Higher temperatures uniformly increase this. Trendelenburg (8) showed that lowering the temperature decreased the effect of strophanthin on perfused frogs' hearts. The difference between given changes in temperature he found to be greater with more concentrated solutions and at the lower temperatures. Gunn (9) found that the effect of strophanthin on perfused rabbits' hearts was more prompt at higher temperatures and thought that this was due to the increased coronary circulation and therefore greater exposure of the heart to the drug. Sollmann, Mendenhall, and Stingel (10) found that the toxicity of ouabain for intact frogs or their isolated hearts increased with the temperature, that this increase was greater per degree at the lower than at the higher temperatures, and that the increase was greater than could be accounted for by the increase in heart rate, which in itself enhances the action of the drug. Weizsäcker (11), Clark (12), and Issekutz (7) noted a more rapid action of the digitalis bodies on frogs' hearts when the rate of contraction as controlled by single induction shocks was increased. Weizsäcker (5) distinguished between the time it took for digitalin to show its first effect on the heart by an increase in the strength of the contractions (*Giftbindung*) and the time to produce standstill (*Giftwirkung*), and observed that the ventricular rate influenced the latter but not the former. Pittenger and Vanderkleed (13),

working with the effects of tincture and fluid extract of digitalis on gold fish, found that although the weight of the fish was not important in determining the time to produce death by a certain concentration of digitalis, changes in temperature made striking differences.

Influence of the General Condition of the Heart and of the Pressure of the Perfusion Fluid.—The general condition of the heart greatly influences the effect produced on it by a given amount of digitalis. Clark (12) showed that strophanthin had a more marked and more rapid effect on perfused hearts if the frog was previously in poor health, or if the heart had been long perfused. Williams (14) and later Krailsheimer (15) found that the degree of pressure to which the frog's heart is subjected in perfusion experiments affects the action of digitalin and helleborein. Clinically it is known that although the mechanism of action of the drug may be the same, the influence of digitalis on diseased hearts may result in a different effect than on normal hearts.

Influence of the Character of the Perfusion Fluid on the Type of Standstill.—There has been some difference of opinion as to the factors that determine the position (systole or diastole) in which a heart will stop under the intoxication of digitalis. The location where the solution is applied is important in this connection. Jacobj (16) and Wybauw (17) found that if the digitalis substance is applied to the outside diastolic standstill results, while if it is applied to the inside systolic arrest occurs. The use of blood serum in the perfusion fluid also alters the subsequent effects of digitalis. Cushny and Gunn (18), for instance, observed that the addition either of homologous or heterologous serum to Ringer solution in perfusing rabbits' hearts caused first a stage of stimulation and later a period of depression in the strength of contraction. The hearts could be restored to a normal condition by returning to the use of Ringer fluid. Gröber (19), experimenting with strophanthidin obtained by Fraser (20) from *Strophanthus hispidus*, noticed that the amount of the drug used influenced the result, for whereas 0.8 mg. in 1,000 cc. of perfusion fluid arrested a cat's heart in systole, 0.5 mg. stopped it in diastole. The perfusion fluid consisted of one part of defibrinated calf blood and two parts of Ringer solution. Clark (12) found that dilute concentrations are apt to produce diastolic arrest, and more concentrated ones, systolic arrest. The effect is more marked if the heart is already in a hypodynamic condition, due to prolonged perfusion. Werschinin (21), using Williams' (14) apparatus, concluded that 0.05 mg. of strophanthin (Boehringer) in 50 cc. of Ringer solution or normal saline, arrested frogs' hearts in systole, but lower dilutions in diastole. He added that the presence of one-third the volume of rabbit blood cells or serum would enable the lower dilutions to produce systolic arrest. In this work doubt remains as to whether the pressure in the perfusion system might not have played a part in producing diastolic standstill. Clark (22), on the contrary, found that defibrinated frog blood distinctly diminished the activity of strophanthin on perfused hearts, while the serum alone had no such effect. He thought that the red blood cells absorbed small amounts of the drug.

Influence of Inorganic Salts.—The inorganic salts influence the effects produced by digitalis. A perfusion fluid not containing calcium requires twice the amount of digitoxin to produce standstill that is necessary with the ordinary amount of calcium, according to Clark (6). He also found that the digitalis effect is slightly increased by the absence of potassium salts. Conversely, Burridge (23) found that the presence of digitoxin increased the sensitiveness of the heart to calcium.

Fate of Digitalis Bodies in the Organism.—The study of the fate of digitalis glucosides within the body, *i.e.* their combination, destruction, and the method of their excretion, is still incomplete. Cloetta and Fischer (24) gave 1 mg. of digitoxin to each of ten frogs; at the end of 1 hour all the hearts were arrested. In extracting the ten hearts no digitoxin was found by a method that could detect 0.04 mg. None was found in the hearts of twenty additional frogs although an emulsion of the rest of the bodies, excluding the skins, yielded one-third of the digitoxin injected. Nor was any found in the hearts of rabbits and dogs killed with the drug. But when emulsions of heart muscle were incubated with digitoxin at 37°C. for 4 hours, twice as much digitoxin could be recovered from the sediment, that is from the heart tissue, as from the supernatant fluid. On incubating liver a similar result was noticed. Incubations of this nature therefore did not destroy the drug, for practically the entire amount of digitoxin could be recovered from dog's heart muscle, liver, or brain. In similar incubation experiments of frog's heart, muscle, and liver, Clark (22) likewise found that strophanthin was neither destroyed nor combined with unincubated tissue. The behavior of digitalis bodies under the influence of heart muscle in the living animal and of incubated heart muscle accordingly differs. Cloetta and Fischer also examined the urine and noticed that varying amounts of the drug could be detected; in one experiment, 0.3 mg. of digitoxin, for instance, was recovered 4 hours after 3 mg. had been injected into the vein of a rabbit; in another experiment, 0.8 mg. was found in the urine during 4 days on which 4.9 mg. were injected subcutaneously. In still another experiment, 1 hour and 20 minutes following a large intravenous dose of 5 mg. to a dog, they found none in the heart, 0.8 mg. in the liver, and 3 mg. in the entire blood. Clark (22) in his experiments found part of the injected drug in the blood. He gave 0.02 mg. of strophanthin to each of six frogs subcutaneously; all died in ½ hour, and he found about 0.01 mg., or 8 per cent, in the entire blood. In what physical or chemical state it circulates in the blood is not known, but that it is active has been proved by experiment. Clark (22) has shown that small amounts of strophanthin could be found in the blood of grass snakes 16 hours after subcutaneous injection. The whole blood of frogs, he found, diminished the action of strophanthin. Serum alone or washed corpuscles alone had no such effect. He thought that the red blood cells absorb strophanthin in small amounts but only in the presence of serum. Rabbit red cells, on the other hand, do not absorb strophanthin. But in the absence of satisfactory methods for detecting the digitalis bodies, their

presence and the duration of their action cannot be accurately known. Hatcher (25) tested out nine cats and found that after intravenous injection of 0.3 mg. of ouabain, one showed 32 per cent in the blood stream, one 45 per cent, and seven none at all. Similarly after the injection of 5 mg. of digitoxin in two different cats, none was found in the blood in 4 and 5 minutes.

These experiments show that part of the drug remains in the blood stream for a while at least, that a portion is excreted unchanged by the kidney, and that the fate of the rest is uncertain. The heart, therefore, appears either to utilize very small amounts, or to render the drug unrecognizable after it has been acted upon. Hatcher (26) made similar studies and found that in alcoholic extracts of all the tissue of the rat made 5 minutes after subcutaneous injection, strophanthin could be recovered almost entirely. But if the rat was allowed to live a day, only a portion could be found in the gastrointestinal tract, and none in the other tissues. The tissues of the rat, which are highly tolerant to digitalis, destroy the drug, and part of the drug is reexcreted into the gastrointestinal tract. Hatcher and Bailey (27) also showed that in the duodenal loop of a dog, without producing toxic effects, an amount of strophanthin could be destroyed equal to 10 times the minimum lethal dose administered intravenously. This then is the evidence in favor of the view that animal tissue destroys digitalis bodies.

Perfused Hearts.—The experiments of Cloetta and Fischer, Clark, and Hatcher accordingly show that digitalis substances cannot be recovered from living heart muscle, but can be found if the muscle is incubated. Some indication of the amount of digitalis that is taken up and destroyed by the heart in producing standstill may be obtained from perfusion experiments. Straub (1) perfused the heart of a number of frogs (average weight 68 gm.) with the same solution until it was arrested. Although unable to extract any digitalis from the poisoned hearts, he concluded that a certain amount, probably less than 0.0002 mg., was used up. Clark (6) by a similar technique determined that each frog's heart used up about 0.0013 mg. of digitoxin and 0.00008 mg. of strophanthin. Grünwald (3) and Weizsäcker (5) found that to accomplish this result the equivalent amounts of digitalin were 0.05 to 0.1 mg. Weizsäcker (5) made the important observation that the amount of digitalin that combines with the heart is the same, whether the heart is arrested rapidly by a concentrated solution, or more slowly by a weaker solution. If amounts no larger than these suffice to arrest the heart, the amount of the poison in the perfusion fluid must be several times greater than the amount that is taken up by the heart; *i.e.*, in order to be lethal the minimum amount of strophanthin contained in 2 cc. of fluid must be 0.001 mg. But of this amount of strophanthin, the heart, as far as the experiments show anything, takes up somewhere between 0.0002 mg. and 0.00008 mg. (average 0.00014 mg.). In other words, a much greater amount is necessary than is actually made use of.

Issekutz (7) calculated the concentration of strophanthin within the heart by weighing the heart and determining how much strophanthin was lost from the

perfusion fluid. He found in this way that the concentration was 40 times as great inside the heart as in the perfusion fluid and 15 to 20 times as great for digitoxin. Weizsäcker (5) had previously calculated in a similar way that the concentration of strophanthin within the heart muscle was just equal to that in the fluid, while that of digitalin was 25 times as great within the heart muscle as in the perfusion fluid. But careful analysis of his figures shows an error in his calculation for he did not compare the lowest dilution of strophanthin in the perfusion fluid that can cause standstill with the concentration in the heart. This would bring the figure to about 20 and would then agree with the findings of Issekutz. It must be recognized in this connection that when a diminution of the drug is found in the perfusion fluid, it need not be in the heart, as has been mentioned above. It is more probable that it is destroyed by the heart and is no longer recognizable, and that the concentration of the active principle in the perfusion fluid is slightly diminished but always greater than in the heart.

There are facts that point to a selective action of the digitalis bodies on the heart, although other body tissues seem to be affected by the drug. Clark (22) found, for instance, that 0.0005 mg. per cc. of strophanthin was required to produce standstill of the heart, 0.1 mg. per cc. to produce vasoconstriction of the leg or contraction of an isolated stomach ring, and 1 mg. to kill voluntary muscle. And Schliomensun (28) found that alcoholic phosphatids, which possess an especial capacity for combining chemically with the digitalins, could be isolated from the hearts of the human cadaver and of the dog while the corresponding fractions obtained from the skeletal muscles had no such affinity.

Action by Physical or Chemical Processes.—The apparent dependence of these drugs on their concentration has led to the view that their mode of action is a physical one at the surface of the muscle. There are facts, however, that point rather to a chemical change in the heart. Straub (1) showed in perfusion experiments that, if strophanthin solutions were removed and the heart was washed, it revived; recovery did not take place if washing was delayed too long. In the latter event the action was not reversible; *i.e.*, the action of the drug is physical to a certain point only. Issekutz (7) similarly found that the heart frequently could not be revived, even if washed before showing a toxic effect. Clark (6) perfused frogs' hearts with 3 cc. of a solution containing 0.01 mg. of digitoxin. He found, like Straub, that if the heart was washed in 8 minutes, it died subsequently in systole, but if washed in 5 minutes, standstill was prevented

The analogies which have been made use of in this discussion are numerous. It is impossible, however, to interchange apparent equivalents in a problem as complex as that of the action of the digitalis glucosides. The following facts illustrate this point. The subcutaneous minimum lethal dose of strophanthin is 30 times greater for a rat than for a rabbit and 20 to 40 times the concentration is needed in the perfusion fluid to arrest the isolated heart. On the other hand, while the intact grass snake is 30 times more tolerant to a subcutaneous injection of strophanthin than is the frog, the isolated heart is 1,000 times more

tolerant. Another instance in which simple substitution would lead to error occurs in determining the relative toxicity of the substances for a certain concentration and inferring that the same relation holds true for other concentrations. Issekutz (7), for instance, shows that 5 cc. of 0.1 per cent digipuratum is equivalent to 0.001 per cent strophanthin, *i.e.* a ratio of 1: 100, but 5 cc. of 0.03 per cent digipuratum is equivalent to 0.0000625 per cent strophanthin, a ratio of 1: 500.

The foregoing review indicates how difficult it is to resolve some of these factors. The more important ones are temperature, rate, the general condition of the heart, the pressure of the perfusion system, and the organic and inorganic constituents in perfusion fluids. Animals differ widely; and their reactions are not constant when the mode of administration is changed or when different glucosides are compared. The experiments often yield contradictory results. Careful analyses sometimes uncover the cause. Occasionally, however, the lack of harmony cannot be explained. The absence of a chemical test for digitalis renders it impossible to make any but general statements about excretion, destruction, and combination of the drug by the living organism. But this review indicates in part wherein the difficulties of the digitalis problem lie. They are comprised principally in the diversity of the methods employed, and in the failure to appreciate that results obtained so variously can scarcely serve as the basis of satisfactory generalization. There is no intention to obscure the value in themselves of the important contributions which have so far been made. But with the means now at our disposal and as the result of the experience now accumulated it becomes important that certain requirements be observed in the plan of the experiments in order that the results may be useful in laying the foundations for clinical practice. In the experiments now reported cats have accordingly been employed because of their relatively uniform reaction to the digitalis bodies and because the same animal can be used for a number of separate experiments. Repeated use in this way made it possible to avoid the danger of lack of uniform results due to varying susceptibility even in animals of the same species. The technique of electrocardiographic registration permitted the performance of the experiment so that operative procedures on the heart itself were not undertaken and disturbances from this source were avoided. This technique in addition afforded the opportunity to study in detail the influence of time on the results of administration.

Method.

Cats were selected in preference to other laboratory animals because their reaction to digitalis is more like that of the human subject. Crystalline g-strophanthin was used because of its chemical purity and to avoid the confusion from interaction of the principal glucosides if the whole digitalis leaf is used. A 1 per cent alcoholic solution was prepared and kept, and from this a 0.001 per

cent solution was made up every few days. This was used in the intravenous injections. There was no indication that the stock solution deteriorated during the period of the experiments. The solutions were kept sterile. The cats were etherized during the operative procedure. Afterward as little ether was used as was necessary to keep the animal sufficiently quiet for taking satisfactory electrocardiograms. But in consequence cats were occasionally lost by an overdose of ether, although it was always possible to distinguish by the electrocardiograms an ether death from one produced by strophanthin. During some experiments no additional ether was given after the injections were started. The strophanthin solution flowed from a graduated burette into the right saphenous vein.

Use of the Electrocardiograph to Detect the Toxic Effect.—The string of the galvanometer (Edelmann model) was connected with the right fore and left hind leg (Lead II). Electrocardiograms were taken before the injection of the drug and frequently during the course of the experiment. It was observed by Halsey (29) that if 40 to 60 per cent of the minimum lethal dose[1] of strophanthin is given intravenously extrasystoles appear. He fixed no time interval during which he gave strophanthin, although he states that 30 per cent was given in about 15 minutes. He gave the rest at irregular intervals. It is, in fact, impossible to find in any previous work determinations of what portion of the minimum lethal dose, given in a definite interval of time, is required to produce extrasystoles. The smallest amount required to cause ectopic premature beats we propose to call the "minimum toxic dose." The only reliable method of determining the minimum toxic dose is electrocardiographic. This method excludes the possibility of misinterpreting the nature of the irregularity. It also dispenses with the necessity of exposing the heart or disturbing the animal. It is

[1] The minimum lethal dose of crystalline strophanthin for cats as determined by Hatcher and Brody (Hatcher, R. A., and Brody, J. G., *Am. J. Pharm.*, 1910, lxxxii, 360) is 0.1 mg. per kilo of body weight if given intravenously and almost continuously over a period of $1\frac{1}{2}$ hours. This was confirmed by Jamieson (Jamieson, R. A., *J. Exp. Med.*, 1915, xxii, 629), who used the same active principle as that in the experiments reported here.

more reliable to use the occurrence of ventricular ectopic beats as the index of toxic action than the vagal effects, such as slowing of the heart rate, lengthening of the auriculoventricular (P R) interval, or the development of exaggerated sinus arrhythmia.[2] Electrocardiograms of typical toxic reaction with subsequent death in ventricular fibrillation are shown in Fig. 1. In a very few instances the toxic effect was similar to those described by Cohn (30) and Christian (31) as resulting from digitalis intoxication in man. The form of the ventricular complex continually changes: an example of this reaction is shown in Fig. 2. Injections were made every 6 minutes and frequently, on approaching the toxic dose, the interval was diminished to 3 minutes. The method of interrupted administration introduces an error of from 3 to 6 per cent in determining the minimum toxic dose, for by giving injections every 6 minutes at the rate of 60 per cent of the minimum lethal dose in an hour, 6 per cent of the minimum lethal dose is given with each injection. The final 6 per cent that is administered, which produces the toxic effect, might have been excessive by 5 per cent, for an additional 1 per cent might have produced the same toxic effect. This error cannot be avoided by giving an absolutely continuous injection, for in that case, the amount of drug given during the last few minutes before the toxic effect appears might have been unnecessary.

Frequency of Spontaneous Nodal Rhythm in Cats.—In the interpretation of cardiac irregularities in cats, attention is called to the great frequency of spontaneous nodal rhythm (Fig. 3). It would be almost impossible to recognize this alteration in the heart's mechanism by any means other than electrocardiographic. Of seventeen animals (Table I), nine showed nodal rhythm one or more times during the various experiments. Some cats showed it several times, others only once or twice. Eight showed it before strophanthin was given in that particular experiment. Some of them also showed it during the early part of the administration, when very small amounts had been given, one after 8 per cent of

[2] Although more reliable, we do not recommend the occurrence of ventricular ectopic beats as the sign of toxic action in clinical medicine, as earlier signs are available.

TABLE I.

Experiment No.	Date.	Weight.	Rate of injection.		Minimum toxic dose (amount to produce toxic effect).	Per cent of minimum lethal dose required to produce effect calculated from.		Time for effect.	Type of effect.*	Spontaneous irregularity
			Per cent of minimum lethal dose.	Time.		Original weight.	Actual weight.			
	1917	*kg.*			*cc.*					
1 a	Jan. 8	3 00	40	1 hr.	Leak in cannula.	?	?	104 min.	V. E. C.	
b	" 10	2.65	40	2 hrs.	13 8	46	52	133 "	"	N. R.
c	" 12	2 40	40	½ hr.	8 4	28	35	23 "	"	
d	" 16	2 05	40	¼ "	15 2	50	74	20 "	"	
2 a	Jan. 8	4.05	40	1 hr.	Leak in cannula.	?	?	134 min.	V. E. C.	
b	" 11	3 65	40	2 hrs.	16.0	40	43	117 "	F. P. V. C.	
c	" 12	3.50	40	½ hr.	16 0	40	46	.28 "	"	
d	" 16	3.45	40	¼ "	25.5	62	70	24 "	V. E. C.	N. R.
3 a	Jan. 9	3.35	40	1 hr.	13 4	40	40	56 min.	V. E. C.	N. R.
b	" 11	3.35	40	2 hrs.	15.6	47	47	141 "	"	"
4 a	Jan. 16	3.65	40	1 hr.	22.0	60	60	95 min.	F. P. V. C.	N. R.
b	" 20	3.40	40	¼ "	16.8	46	49	20 "	C. V. C.	"
5 a	Jan. 18	3 00	40	1 hr.	18 0	60	60	88 min.	V. E. C.	
b	" 22	2.70	40	¼ "	19.2	64	71	23 "	"	
c	" 26	2.65	40	2 hrs.	12.0	40	45	117 "	F. P. V. C.	N. R.
6 a	Jan. 23	3.00	50	1 min.	15.0	50	50	13 min.	Tracings lost.	
b	" 29	2.80	50	1 hr.	10 5	35	38	38 "	C. V. C.	
c	Feb. 2	2.35	50	2 hrs.	9 0	30	38	70 "	F. P. V. C.	
7 a	Jan. 24	2.25	60	1 min.	13.5	60	60	∞	0	
b	" 30	2.00	60	1 hr.	11.7	52	59	49 min.	F. P. V. C.	
c	Feb. 5	1.65	52	1 min.	11.7	52	71	12 "	C. V. C.	

8 a	Jan. 25	2.95	60	1 hr.	18.5	63	63	76 min.	F. P. V. C.	
b	" 30	2.50	60	¼ "	15.2	52	61	13 "	V. E. C.	
c	Feb. 6	2.00	60	2 hrs.	11.7	40	59	96 "	F. P. V. C.	N. R.
9 a	Feb. 7	4.40	60	¼ hr.	19.3	44	44	11 min.	C. V. C.	
b	" 13	3.45	60	2 hrs.	20.8	47	60	91 "	O. P. V. C.	Bigeminy.
c	" 19	3.15	60	1 hr.	15.6	35	49	35 "	V. E. C.	
d	" 23	2.90	60	4 hrs.	18.9	43	65	173 "	F. P. V. C.	
10 a	Feb. 8	3.20	60	2 hrs.	23.0	72	72	143 min.	F. P. V. C.	
b	" 13	2.90	60	½ hr.	22.8	71	78	36 "	?	
11 a	Feb. 9	3.25	60	2 hrs.	23.3	72	72	143 min.	O. P. V. C.	
b	" 14	2.90	60	½ hr.	20.6	64	71	35 "	V. E. C.	
12 a	Feb. 14	3.00	60	¼ hr.	14.4	48	48	9 min.	V. E. C.	N. R.
b	" 20	2.45	60	½ "	10.8	36	45	17 "	"	"
c	" 24	2.35	60	1 "	12.6	42	54	41 "	"	"
d	" 28	2.15	60	4 hrs.	10.8	36	50	122 "	"	"
13 a	Feb. 15	2.65	60	1 hr.	6.4	24	24	23 min.	O. P. V. C.	
b	" 20	2.25	60	¼ "	6.4	24	28	5 "	"	N. R.
14 a	Feb. 16	3.40	60	4 hrs.	18.0	53	53	214 min.	F. P. V. C.	
b	" 21	2.95	60	1 hr.	18.0	53	61	48 "	V. E. C.	N. R.
c	" 26	2.50	60	¼ "	21.3	63	85	17 "	F. P. V. C.	
15 a	Mar. 2	4.70	35	2 min.	16.4	35	35	16 min.	V. E. C.	
b	" 7	4.55	60	1 hr.	16.8	36	37	38 "	F. P. V. C.	
16 a	Mar. 5	3.15	30	1 min.	13.1	42	42	12 min.	C. V. C.	
			60	2 hrs.						
b	" 9	2.75	30	1 hr.	15.2	48	55	45 "	F. P. V. C.	
17 a	Mar. 8	2.85	30	1 min.	14.4	51	51	83 min.	V. E. C.	
			60	2 hrs.						
b	" 19	2.15	30	1 min.	11.0	30	45	9 "	F. P. V. C.	

* V. E. C. indicates ventricular ectopic tachycardia; F. P. V. C., frequent premature ventricular contractions; O. P. V. C., occasional premature ventricular contractions; C. V. C., changing ventricular complexes; N. R., nodal rhythm.

the minimum lethal dose had been injected. Two others, No. 9 b and one not included in Table I, showed spontaneous bigeminy, every other beat being a premature ventricular systole, before any strophanthin was given. Three cats had nodal rhythm in the first experiments (marked "a" in Table I) on the respective animals. There is no reason to believe that the cats had taken any drug that might have caused this rhythm. None of them had previously been experimented upon. The high incidence of spontaneous nodal rhythm is pointed out because it may have a bearing on experiments concerned with this arrhythmia as in the work of Lewis, White, and Meakins (32).

Independence of the Minimum Toxic Dose of the Speed of Administration of the Drug.—In the experiments to be described we compare the effects of rapid with slow administration in the same animal. The method is designed to yield information on the problem of whether strophanthin acts by means of its concentration or by virtue of the total amount present. The volume of the blood remains constant. By injecting it rapidly strophanthin is distributed in the circulation quickly, and the heart, therefore, is immediately exposed to a high concentration; when the injections are slow, the heart is exposed to a lower concentration for a given length of time, for a smaller amount of the active principle is contained in the same volume of blood. Thus the rate of injection determines the concentration in the blood stream. Some cats served for four separate experiments, others for three, and some for only two. The method of administration was changed from cat to cat; some received the rapid injection first and later the slow one. The reverse method was employed in others. The original minimum lethal dose was calculated on the original weight, and in later experiments the same dose was used, although the weight had diminished. This will be discussed below. In this way one could determine whether the animal's later condition as a result of the action of the drug, or the long experimentation, or the administration of ether, altered the amount of the drug required to produce the same toxic effect. There was no indication that any striking change in the animal's susceptibility to the drug took place. In the first experiments the solution (0.001 per cent)

was given at such a rate that 40 per cent of the minimum lethal dose was injected in an hour. The same cat, usually 4, though sometimes more and occasionally fewer days later, when free of the drug was subjected to the same procedure but the rate of injection was 40 per cent in 2 hours, or 40 per cent in $\frac{1}{2}$ hour, etc. The cat was considered free from the effects of the drug because Hatcher (33) showed that 24 to 48 hours following a toxic but nonlethal intravenous injection of strophanthin, a similar dose is required to produce a similar result. In the later experiments 60 per cent of the minimum lethal dose was given, because it caused toxic effects more regularly. The injections were stopped when the toxic effect occurred. This could usually be detected by watching the shadow of the galvanometer string, because of the abnormal form of the ectopic beat, and the occurrence of an irregularity. Occasionally it was difficult to be certain whether a toxic effect was produced; then electrocardiograms were taken frequently. When the films were developed, the intoxicating dose was estimated.

EXPERIMENTAL.

The experiments are divided into three groups: those in which was studied the influence of the speed of injection on the amount of strophanthin required to produce a toxic effect; those in which single injections were made to obtain the minimum toxic and lethal dose and to compare this with the amount required when the rate of injection is slow; and transfusion experiments made to obtain additional information on the concentration of the drug in the blood.

Group 1.—Group 1 (Table I) contains seventeen experiments. In these was studied the influence of the speed of injection on the amount of strophanthin required to produce a toxic effect. The animals frequently lost weight. Whether this resulted from loss of appetite, or from prolonged etherization and the operative procedure, or from strophanthin intoxication is not known. The animal usually ate nothing after the operation and very little the next day. On the 4th day it was usually operated upon again. The loss in weight was disturbing in calculating the minimum lethal

dose. It seemed correct, however, to base the minimum lethal dose on the original, rather than on the actual weight (both of which were ascertained), for it is not likely that the heart lost weight during these experiments as rapidly as the whole body.[3]

There are (Table I) distinct individual variations among cats in susceptibility to toxic doses of strophanthin. It required, for instance, 72 per cent of the minimum lethal dose, given at the rate of 60 per cent in 2 hours, to produce toxic effects in Cats 10 a and 11 a, while only 24 per cent, at the rate of 60 per cent in 1 hour, was required in Cat 13 a. The difference in rate of administration will be shown not to influence the minimum toxic dose. Other animals required amounts between these limits. The significant point is that in the second, third, and fourth injections in the same animal, although slight variations in the minimum toxic dose occur, they do not depend on the speed of administration. This conclusion is warranted from the average of the experiments (Table II) and from an analysis of the individual ones.

TABLE II.

Average.	Per cent of minimum lethal dose required to produce toxic effect, calculated from.	
	Original weight.	Actual weight.
Of all in Table I	47.4	53.7
" first injections ("a" in Table I)	51.6	51.6
" all subsequent injections	48.7	54.8
" last injections	46.5	56.9
" 1 hr. first injections	49.4	49.4
" ¼ " injections	50.3	58.9
" ½ " "	47.8	55.0
" 1 " "	45.5	49.5
" 2 hrs. "	45.3	52.0
" 4 " "	44.0	56.0

The average minimum toxic dose of strophanthin in all the experiments (Table II) was 47.4 per cent of the minimum lethal dose,

[3] In a fasting cat the heart lost only 2.6 per cent of its weight, while the liver lost 53.7 per cent, and the whole body over 1,000 gm. (Howell, W. H., A text book of physiology for medical students and physicians, Philadelphia and London, 5th edition, 1913, 914).

if the original weight, and 53.7 per cent if the actual weight before each experiment is used as the basis of calculation. The second figure is higher than the first because of the animal's loss in weight. The average of all first experiments before loss of weight has occurred is 51.6 per cent. The average in the first experiments in which injections were given in an hour is 49.4 per cent. In these the influence of the loss of weight due to experimentation is entirely avoided. On the whole, there are no serious discrepancies in the various averages calculated in Table II. They show that the speed of injection does not influence the minimum toxic dose. The average minimum lethal dose in the short 15 minute experiments was 50.3 per cent; in the longer 4 hour ones 44.0 per cent. In those of intermediate length the percentages increased as one might expect. One might infer from the figures based on the original weights that the slower method requires slightly less strophanthin than the rapid, but the difference is probably not significant. An explanation for it may exist in the fact that in the slower method the dose given just before the toxic effect is produced, has acted a longer time on the heart; while in the rapid experiments an excessive amount was probably injected. The injections, for instance, were made at the rate of 4 cc. every 3 minutes. When the end-point was approached 1 cc. might have been sufficient to cause a toxic effect. Errors of this kind are smaller in the slower experiments because a smaller amount is given every 6 minutes.

Two experiments, Nos. 9 and 12 (Table I), illustrate these points. Four injections at different rates were given in each case. In No. 9 the first injection was given at the rate of 60 per cent in 15 minutes, and the toxic effect (minimum toxic dose) was produced with 44 per cent of the minimum lethal dose. 6 days later the same cat received an injection at the rate of 60 per cent in 2 hours and showed toxic manifestations with 47 per cent; the third injection, 6 days later, was given at the rate of 60 per cent in 1 hour and the minimum toxic dose was 35 per cent; the final one was given 4 days later at the rate of 60 per cent in 4 hours, and the minimum toxic dose was 43 per cent. Similar results were obtained in the four injections in No. 12. The rate at which the

injections were given was also 60 per cent of the minimum lethal dose in varying intervals of time. The variations were 48 per cent for 15 minutes, 36 per cent for 30 minutes, 42 per cent for 1 hour, and 36 per cent for 4 hours. In these instances, then, the minimum toxic dose is independent of the rate of injection. To test the point further, the rate of injection was changed in several instances during the same experiment. If a cat at first was given 30 per cent in 1 minute, injection was continued after waiting 15 minutes at the rate of 60 per cent in 2 hours. The toxic effect again was produced after a certain total amount had been given.

Group 2.—Further evidence on the relation of rate of injection to amount injected was obtained from a series in which large single injections were made in 1 or 2 minutes. It is, of course, impossible in a given animal to forecast the minimum toxic dose.

TABLE III.

Per cent of minimum lethal dose given in 1–2 min.	25	30	35	40	50	55	60	70	75	80	90	100
Result.	4 N.* 1 T.	4 N. 1 T.	1 T.	1 N. 1 T.	2 N. 1 T.	1 T.	1 N. 1 T.	2 T.	1 F.	1 T. 1 F.	1 T. 1 F.	1 T. 2 F.

* N. indicates negative result; T., toxic effect; F., fatal.

It was therefore necessary in a series of animals to obtain it by trial. The averages then served for comparison. There were twenty-nine cats. They received single injections of amounts varying from 25 to 100 per cent of the minimum lethal dose. There was, as was expected, great variation in the minimum toxic dose, for some cats showed effects from 25 per cent, while others required 60 per cent of the minimum lethal dose (Table III). Of five experiments in which 25 per cent was given only one was toxic. One cat in five was found to be intoxicated by injections of 30 per cent; one in two experiments in which 40 per cent was injected, one in three 50 per cent injections, and one in two 60 per cent injections. All injections over 60 per cent of the minimum lethal dose caused toxic effects and many were also fatal. There was also some variation in the minimum lethal dose, for 75 per cent

was fatal to one cat while 100 per cent was not to another. It is difficult to calculate an average toxic dose from such experiments, but in general these figures agree with those of Table II. If flooding the circulation suddenly with a large dose of strophanthin were more toxic than giving the same dose continuously over an hour or two, one would expect a greater percentage of toxic and lethal effects than were obtained. When 30 per cent, for instance, was given, only one of five showed an effect.

These results throw doubt on the conclusion that strophanthin produces its effects in accordance with the concentration of the drug to which the heart is exposed. For the differences in the speed of administration must alter its concentration in the blood stream, the blood volume probably remaining constant. So far, then, the toxic effect appears to be produced when a given total amount has been injected into the blood. An objection to this conclusion may, however, be offered; strophanthin, no matter at what speed administered, may be supposed to remain inert in the blood stream until a certain amount has been given, to provide a concentration sufficient for activity. When the required concentration is reached the heart is intoxicated. The objection is hardly valid, for there is evidence that even before the toxic effects appear strophanthin affects the heart. The therapeutic effects, for instance, of the drug on the heart are obtained by subtoxic doses.

Cohn, Fraser, and Jamieson (34) have shown that the heart muscle may be affected by digitalis before toxic symptoms are produced. Robinson and Wilson (35) show that to bring about T wave changes in the electrocardiogram of cats only 23.9 per cent of the lethal dose is necessary. Gros (36), using very dilute solutions of strophanthin on the perfused frog's heart, produced intermittent diastolic pauses without any other toxic results. These observations suggest that throughout the experiments the cats' hearts take up strophanthin, the toxic symptoms appearing when a certain total amount has been taken up.

Group 3.—Transfusion experiments were undertaken to obtain information on the amount of the drug actually present in the blood.

Experiment 18.—Cat A, weight 4,150 gm. was given 0.1 mg. of strophanthin at 11.05 and 0.1 mg. at 11.15 a.m. At 11.20 ventricular ectopic tachycardia appeared. At 12.50 p.m., 75 cc. of blood were taken from left carotid artery into

citrate solution. From a second cat, B, weight 4,250 gm., (the minimum toxic dose having previously been found to be 66 per cent of the minimum lethal dose), 70 cc. of blood were withdrawn at 12.50 from the left carotid artery. At the same time 70 cc. of strophanthinized blood from Cat A were transfused into the right saphenous vein of Cat B. At 1.10 the injection of strophanthin intravenously was started at the rate of 0.03 mg. every 6 minutes. After 0.12 mg. had been given, ventricular ectopic tachycardia was noticed. The minimum toxic dose equalled 28 per cent of the minimum lethal dose. Death occurred after 0.225 mg. had been given. This equalled 53 per cent of the minimum lethal dose.

The low minimum toxic dose and the low minimum lethal dose indicate that some strophanthin must have been contained in the blood of Cat A, for it took much less additional strophanthin to produce either a toxic or a fatal result in Cat B than we know would otherwise have been required. It is impossible to calculate how much of the drug the transfused blood of Cat A contained, but the result makes it reasonable to say that 1 hour and 45 minutes after the toxic effect had been produced the blood of Cat A contained at least a moderate amount of strophanthin.

Experiment 19.—Cat A, weight 4,400 gm., was given 0.1 mg. of strophanthin at 10.26 and 0.1 mg. at 10.36 a.m. At 10.43 a toxic effect occurred. At 11.55 an added 0.2 mg. was given intravenously. At 12.05 p.m. 100 cc. of blood were taken from the left carotid artery. Then from a second cat, B, weight 3,300 gm. (the minimum toxic dose for which having previously been found to be 67 per cent of the minimum lethal dose), 70 cc. of blood were removed at 12.40. At 12.42, 85 cc. of blood from Cat A were transfused into the left saphenous vein of Cat B. Then at 1.12, 0.026 mg. of strophanthin was given every 3 minutes. A toxic effect was produced with 0.147 mg., the equivalent of 45 per cent of the minimum lethal dose, and death occurred after 0.303 mg. had been injected. This equalled 92 per cent of the minimum lethal dose.

This experiment also indicates that a certain amount of strophanthin was carried over in the blood from Cat A, for the minimum toxic dose for Cat B when first tested was 67 per cent of the minimum lethal dose and after transfusion was only 45 per cent.

Experiment 20.—Cat A, weight 2,900 gm., was given on Mar. 31 at 10.35 a.m., 0.1 mg. of strophanthin; at 10.53, 0.05 mg.; at 10.57 a toxic effect was observed. At 11.40, 70 cc. of blood were taken from the left femoral artery. At 11.45, 75 cc. of normal saline solution were given. At 12 m. 25 cc. of citrated blood from Cat B were injected; at 12.37 p.m., 0.05 mg. of strophanthin was given; at 12.42, 0.05 mg., and at 12.47, 0.02 mg. The fatal dose was 0.27 mg.; *i.e.*, 93 per cent of the minimum lethal dose.

On Mar. 27, the minimum toxic dose of Cat B, weight 2,600 gm. was found to be 77 per cent of the minimum lethal dose On Mar. 31, at 11.45. 50 cc of blood were taken from the right carotid artery At 11.52, 65 cc of strophanthinized blood from Cat A were transfused into Cat B At 1.17, 0.063 mg. and at

1.23 an additional 0.063 mg. of strophanthin were injected. At 1.45 a toxic effect was produced. In this experiment, therefore, a toxic effect was produced with 48 per cent of the minimum lethal dose. At 1.48, 0.031 mg., and at 1.51, 0.031 mg. of strophanthin were added. The cat died at 1.57 after taking 72 per cent of the minimum lethal dose.

Cat A, weight 2,900 gm.		Cat B, weight 2,600 gm. (minimum toxic dose previously found to be 77 per cent of minimum lethal dose).	
a.m.-p.m.		*a.m.-p.m.*	
10.35	0.1 mg. of strophanthin.		
10.53	0.05 " " "		
10.57	Toxic effect.		
11.40	Bled 70 cc.		
11.45	75 cc. of saline solution intravenously.	11.45	Bled 50 cc.
		11.52	65 cc. of strophanthinized blood of Cat A.
12.00	25 cc. of blood from Cat B.		
12.37	0.05 mg. of strophanthin.		
12.42	0.05 " " "		
12.47	0.02 " " "		
12.51	Died.		
		1.17	0.063 mg. of strophanthin.
		1.23	0.063 " " "
		1.45	Toxic effect.
		1.48	0.031 mg. of strophanthin.
		1.51	0.031 " " "
		1.57	Died.

Minimum toxic dose for Cat A = 52 per cent of the minimum lethal dose. Lethal dose for Cat A = 93 per cent of the minimum lethal dose. Minimum toxic dose for Cat B = 65 cc. of strophanthinized blood of Cat A plus 48 per cent of the minimum lethal dose. Minimum lethal dose for Cat B = 65 cc. of strophanthinized blood of Cat A plus 72 per cent of the minimum lethal dose.

From Cat A, then, 43 minutes after the toxic effect was produced about half[4] of the blood was removed (70 cc.). Some strophanthin must have been removed with the blood; however, 93 per cent of the minimum lethal dose was required to cause death. Cat B, which required 77 per cent of the minimum lethal dose to produce toxic effects in the preliminary test, was toxic after the injection of the 65 cc. of blood taken from Cat A, plus 48 per cent of the minimum lethal dose. It died after 72 per cent was injected. This amount was less than the minimum toxic dose on the first occasion.

[4] Calculated on the basis that 5.5 per cent of cat's weight is blood. (Ellenberger, W., and Scheunert, A., Lehrbuch der vergleichenden Physiologie der Haussäugetiere, Berlin, 1910.)

Experiment 21.—The minimum toxic dose of Cat A, weight 3,850 gm., was found to be 72 per cent of the minimum lethal dose. On Apr. 3, at 11 a.m., 0.092 mg. of strophanthin was injected, and at 11.15 an additional 0.08 mg. (47 per cent). No toxic effect was produced. At 12.42 p.m., 90 cc. of blood were withdrawn from the left femoral artery. At 12.46, 45 cc. of blood taken from a normal cat were transfused. At 12.55, 25 cc. of normal saline solution were given. Beginning at 1.06, 0.046 mg. of strophanthin was given every 6 minutes until death. The toxic effect occurred after an additional 34 per cent of the minimum lethal dose (total 81 per cent) and the lethal dose after 116 per cent was injected.

This experiment shows in a somewhat different manner from the previous transfusion experiments that an appreciable amount of strophanthin must have been removed in bleeding, for although 47 per cent of the minimum lethal dose was given without producing a toxic effect, 34 per cent more had to be given after the bleeding to cause the toxic symptoms. If all the strophanthin had been bound either to the heart or to the other tissues so much would hardly have been required. The fatal dose was in excess of the expected amount if all had been retained in the body. Of the four transfusion experiments No. 21 was the only one which showed a larger minimum toxic dose than was previously found in the corresponding cat, and it was also the only one in which the minimum lethal dose was more than 100 per cent.

These results are in accord with the idea that strophanthin remains in the blood stream, for while in Experiments 18 to 20, strophanthinized blood from other cats was added, in Experiment 21 strophanthinized blood was removed. These transfusion experiments are imperfect and do not permit an accurate calculation of the concentration of the drug in the blood stream. They show, however, that an appreciable amount is present in the peripheral circulation an hour or more after intravenous administration, whether a toxic effect is produced or not.

Strophanthin almost invariably caused death by fibrillation of the ventricles; respiration continued longer. There is no way of preventing this or of stopping it after its inception. If a fatal issue depends on the presence of strophanthin in the blood, the withdrawal of blood should prevent or delay death. An attempt was accordingly made after injecting 100 per cent of the minimum lethal dose to prevent ventricular fibrillation by bleeding the animals as soon as the toxic effect was observed. This procedure failed in three experiments. Whether a fatal outcome would

occur if the drug were given slowly was not ascertained. Ether never caused fibrillation of the ventricles; respiration stopped, but the electrocardiogram showed the presence of heart block for a long time. Massaging the heart when the ventricles fibrillate, frequently delayed death for ½ hour. The pupils remained small, and respiration continued. If massage was stopped the pupils dilated and respirations became labored or ceased; on resuming massage the pupils again became small, and the respirations more easy. In the absence of any other treatment massage of the heart offers a possible method of treating fibrillation, although the chances are against a favorable outcome. In all but one instance the cat finally died.

The outcome of this exceptional case, Cat 7 b, is important, for no other case of recovery from digitalis poisoning in an experimental animal has been recorded. This cat was given 0.013 mg. of strophanthin every 6 minutes; the ether cone was kept in place but no ether was added after the injections began. In 49 minutes, when 52 per cent of the minimum lethal dose had been given, extrasystoles were observed (Fig. 4); the injections were stopped, but 10 minutes later the ventricles fibrillated. Electrocardiograms were obtained at frequent intervals during this time. The heart was massaged without opening the chest. For a time the galvanometer string was practically motionless, then unusual deflections were seen, and later a return to typical ventricular fibrillation; finally ventricular tachycardia developed. The breathing improved; the pupils, which were at times markedly dilated, became small, and the cat recovered. The last tracing (Fig. 4), taken 22 minutes after the onset of ventricular fibrillation, shows a normal cardiac mechanism except for delayed conduction (P–R) time, 0.16 second. 6 days later the animal was used again for an experiment.

A number of details deserve notice. (1) In one experiment (No. 20) ventricular ectopic tachycardia did not appear until 90 per cent of the minimum lethal dose had been injected. The warning is therefore expressed that intoxication may be present, without the appearance of extrasystoles.

(2) In fractional administration irregularities usually occurred ½ to 4 minutes after the last injection. Their duration was usually longer than 30 minutes. Durations of 30 and 50 minutes were observed in two cats, and in seven other cats, of ½ to 4 hours. After single large doses, irregularities began in 2 to 15 minutes. Death occurred in 9 to 30 minutes.

(3) Numerous fatalities in patients have resulted from the intravenous administration of strophanthin, but most of them have occurred when this drug was given to patients who recently had taken digitalis, or when large doses were repeated on the same day. It is

TABLE IV.

Experiment No.	Method of administration.	Minimum toxic dose.	Minimum lethal dose.	Margin of safety.	
		per cent	*per cent*	*per cent*	
3 b	40 per cent of minimum lethal dose in 2 hrs.	47	70	23	(23)*
22 c	40 per cent in 1 min. was toxic; 2½ hrs. later irregularity persisted and 20 per cent was fatal	40	60	20	(25)
5 c	40 per cent of minimum lethal dose in 2 hrs.	40	78	38	(43)
6 c	50 per cent of minimum lethal dose in 2 hrs.	30	45	15	(19)
7 c	52 per cent in 1 min. was toxic; 1 hr., 20 min. later irregularity had disappeared and 20 per cent in 1 min. was fatal	52	72	20	(27)
8 c	60 per cent in 2 hrs.	40	55	15	(22)
23 a	70 per cent in 2 min. was toxic; 1 hr., 50 min. later heart again regular and 30 per cent in 1 min. was fatal	70	100	30	(30)
10 b	60 per cent in ½ hr.	71	89	18	(20)
12 d	60 per cent in 4 hrs.; waited 1 hr. after toxic effect; heart irregular then; 6 per cent in 1 min. was fatal	36	42	6	(8)
20 a	60 per cent in ½ hr.	54	96	42	(42)
21 a	60 " " " ½ "	72	90	18	(18)

* All percentages were calculated on the basis of the original weight of the animal. The figures in parentheses are calculated on the actual weight and are slightly higher.

impossible from these experiments to make recommendations for the administration of strophanthin to man. They show that a margin of safety exists, represented by the difference between the minimum lethal dose and the minimum toxic dose. This difference (Table IV)

varied between 6 and 42 per cent of the minimum lethal dose. To predict the toxic dose is, therefore, impossible. The dose, for example, that was fatal to Cat 12 was not even toxic for Nos. 3, 10, 20, and 21. To foretell the toxic dose in patients is probably equally difficult. But since these experiments show that the same results can be obtained from slow as from rapid injections, a fractional method of administration suggests itself. If, for instance, 0.5 mg. given in a single injection were fatal, 0.2 mg. might be given followed by 0.1 mg. repeated every half hour until 0.5 mg. is given. It may be possible to regulate the procedure by watching for signs of intoxication, either by auscultating for irregularities or by taking graphic records, to notice changes in the T waves or the P-R time. Under these conditions, giving an excess of more than 0.1 mg. is avoided. Treatment should not be repeated within 24 hours. Vaquez and Lutembacher (37) have reported almost 2,000 intravenous injections of ouabain without harm or fatality.

DISCUSSION AND SUMMARY.

The review of the literature relating to experiments on the action of the digitalis glucosides shows that care must be exercised in estimating the significance of the results published. The perfusion of isolated hearts obviously cannot take into account the question of excretion of the drug or its combination with other tissues. Nor can perfusion experiments take into account the influence of the extracardiac nerves. Vagotomy alone, as Macht and Colson (38) have shown, has a marked influence on the result. And Richards and Wood (39) have shown that the effect of the intravenous injection of strophanthin is altered after section of the splanchnic nerves. Another difficulty in making deductions of a general nature lies in the fact that digitalis bodies differ in their action, in their solubility, and in the rate of their absorption. These difficulties are made clear when the following statements are placed side by side. Strophanthin is much more toxic to a frog's heart than digitonin, but the latter is more toxic to the isolated sartorius (40). Voegtlin and Macht (41), experimenting on isolated coronary arteries, found that digitonin and digalen produced relaxation of the

arteries, while digitoxin and digitalin caused constriction. Fraser (42), many years ago, called attention to the fact that while strophanthin was much more profound in its action on the frog's heart than digitalin, the reverse was true as to their action on the blood vessels.

There is also a marked difference in the reaction and susceptibility of different animals to these drugs. And the problem is further complicated by the change in the mode of administration from animal to animal. The intact grass snake, for instance, is 30 times more tolerant to strophanthin given subcutaneously than is the frog, but the isolated heart is 1,000 times as tolerant. The action of these drugs depends upon the state of the heart, on the temperature of the surrounding media, the proportion of inorganic salts in the fluid, the pressure to which the heart is exposed, the rate of the heart beat, the presence or absence of blood corpuscles or serum, and, no doubt, other factors as well. To obtain identical results under artificial conditions means that all these factors must be controlled. These are requirements which are obviously difficult to meet. The experiments now reported have avoided these difficulties.

The experiments have a bearing on the controversy between Straub and Grünwald. Straub believes that the action of strophanthin is independent of the total amount and depends entirely upon the concentration; Grünwald holds that although the concentration is the prime factor when large quantities of the active principle are used, the total amount is of importance when the dilutions are small and when small amounts are compared. The distinction is important because it is the small amounts and the low dilutions that are employed in clinical medicine. Furthermore, it is not safe to measure the toxicity of digitalis in terms of the time it takes to produce standstill of the ventricles, since Schmiedeberg (43) found that the two factors did not vary directly.

In these experiments, in which crystalline strophanthin was injected intravenously, the amount of the drug needed to produce toxic results as shown electrocardiographically was practically independent of the speed of administration. The use of the same animal for repeated experimentation avoided the error which re-

sults from making comparisons between different species of animals, and eliminated the marked variations due to individual susceptibility. Injections of the drug were made at varying speeds, with intervals of 4 or more days between experiments. The total amount required to produce the toxic effect did not vary significantly. The experiments (Table II) show strikingly that the total amount required to produce a toxic effect is independent of the speed of administration. This conclusion is probable because although the amount was the same, the concentration was considerably greater when 60 per cent of the minimum lethal dose was given in a single dose or in $\frac{1}{4}$ hour than when given in 4 hours. It has been suggested that when the drug is given slowly, it may remain inert in the blood stream and produce a toxic effect only when a proper concentration is reached, this concentration being reached when the required amount has been injected without reference to the speed of administration. Against this view we have noted the fact that the heart is known to be affected by the drug before toxic effects are produced. The transfusion experiments were undertaken to determine whether strophanthin was present in the blood stream. The method showed that strophanthin must be regarded as present and capable of remaining there for a moderate time but proved too crude to give an idea of its actual concentration. A theory of the action of strophanthin may be formulated from the above considerations. It reconciles the conflicting views relating to the importance of concentration and of total amount of the drug. It supposes that the time required to produce a given effect in a heart varies inversely with the concentration of the active principle. The heart utilizes only a small portion (in the neighborhood of 10 per cent) of the drug to which it is exposed no matter what the concentration. A toxic effect results when the heart has taken up a certain total amount of the drug which is a definite small fraction of its own weight. If this theory is correct, it explains why in concentrated solutions the total amount is not important, for the small part that is taken out by the heart does not appreciably alter the concentration, while when very dilute solutions or small quantities are used the amount taken up by the heart diminishes the remaining concentration appreciably;

that is, the "digitalis pressure" becomes lessened. In these experiments the rapid injections forced an adequate amount of strophanthin into the heart rapidly and produced the toxic effect; in the slow injections the same total amount of drug was taken up by the heart only more slowly.

Experiments were carried out on a series of cats in which, after the minimum toxic dose was determined, the injections (some were slow, others more rapid) were continued until death. In this way the margin of safety was determined, the difference between the minimum lethal dose and the minimum toxic dose. It varied from 6 to 42 per cent of the minimum lethal dose.

These experiments have an importance in clinical medicine. They suggest a method for intravenous medication with strophanthin, designed to reduce the danger due to using the drug. After the ventricles have fibrillated, there is no constant way of reviving a heart. Occasionally, as in one experiment, resuscitation may take place after ventricular fibrillation and standstill, by massage of the heart without opening the chest. No opinion can be offered on the practical value of this method.

CONCLUSIONS.

1. Cats vary considerably in their susceptibility to strophanthin and in the extent of the difference between the minimum lethal dose and the minimum toxic dose.

2. The amount of strophanthin necessary to produce a toxic effect in a given cat is independent of the speed of administration to a period of 4 hours. An improvement in the clinical administration of the drug is thereby indicated.

3. A theory of the action of strophanthin is formulated which reconciles the results which point to the importance of the total amount taken up by the heart with those which indicate that the concentration of the drug is the determining factor.

BIBLIOGRAPHY.

1. Straub, W., *Biochem. Z.*, 1910, xxviii, 392.
2. Holste, A., *Arch. exp. Path. u. Pharmakol.*, 1912, lxx, 435.
3. Grünwald, H. F., *Arch. exp. Path. u. Pharmakol.*, 1912, lxviii, 231.

4. Straub, W., *Arch. exp. Path. u. Pharmakol.*, 1913, lxxi, 139.
5. Weizsäcker, V., *Arch. exp. Path. u. Pharmakol.*, 1913, lxxii, 347.
6. Clark, A. J., *Proc. Roy. Soc. Med.*, 1911–12, v, Therap. and pharmacol. sect., 181.
7. Issekutz, *Arch. exp. Path. u. Pharmakol.*, 1915, lxxviii, 155.
8. Trendelenburg, P., *Arch. exp. Path. u. Pharmakol.*, 1909, lxi, 256.
9. Gunn, J. W. C., *J. Pharmacol. and Exp. Therap.*, 1914–15, vi, 39.
10. Sollmann, T., Mendenhall, W. L., and Stingel, J. L., *J. Pharmacol. and Exp. Therap.*, 1914–15, vi, 533.
11. Weizsäcker, V., *Arch. exp. Path. u. Pharmakol.*, 1913, lxxii, 282.
12. Clark, A. J., *J. Pharmacol. and Exp. Therap.*, 1913–14, v, 215.
13. Pittenger, P. S., and Vanderkleed, C. E., *J. Am. Pharm. Assn.*, 1915, iv, 427.
14. Williams, F., *Arch. exp. Path. u. Pharmakol.*, 1881, xiii, 1.
15. Krailsheimer, R., *Arch. exp. Path. u. Pharmakol.*, 1910, lxii, 296.
16. Jacobj, *Arch. exp. Path. u. Pharmakol.*, 1900, xliv, 368.
17. Wybauw, R., *Arch. exp. Path. u. Pharmakol.*, 1900, xliv, 434.
18. Cushny, A. R., and Gunn, J. A., *J. Pharmacol. and Exp. Therap.*, 1913–14, v, 1.
19. Gröber, A., *Arch. exp. Path. u. Pharmakol.*, 1913, lxxii, 317.
20. Fraser, T. R., *Tr. Roy. Soc. Edinburgh*, 1890, xxxv, 955.
21. Werschinin, N., *Arch. exp. Path. u. Pharmakol.*, 1909, lx, 328.
22. Clark, A. J., *J. Pharmacol. and Exp. Therap.*, 1912–13, iv, 399.
23. Burridge, W., *Quart. J. Med.*, 1916, ix, 271.
24. Cloetta, M., and Fischer, H. F., *Arch. exp. Path. u. Pharmakol.*, 1906, liv, 294.
25. Hatcher, R. A., *J. Am. Med. Assn.*, 1913, lxi, 386.
26. Hatcher, R. A., *Am. J. Physiol.*, 1908–09, xxiii, 303.
27. Hatcher, R. A., and Bailey, H. C., *J. Am. Med. Assn.*, 1909, lii, 5.
28. Schliomensun, B., *Arch. exp. Path. u. Pharmakol.*, 1910, lxiii, 294.
29. Halsey, J. T., *J. Exp. Med.*, 1917, xxv, 729.
30. Cohn, A. E., *Heart*, 1913–14, v, 5.
31. Christian, H. A., *Arch. Int. Med.*, 1915, xvi, 341.
32. Lewis, T., White, P. D., and Meakins, J., *Heart*, 1913–14, v, 289.
33. Hatcher, R. A., *Arch. Int. Med.*, 1912, x, 268.
34. Cohn, A. E., Fraser, F. R., and Jamieson, R. A., *J. Exp. Med.*, 1915, xxi, 593.
35. Robinson, G. C., and Wilson, F. N., *J. Pharmacol. and Exp. Therap.*, 1917–18, x, 491.
36. Gros, O., *Arch. exp. Path. u. Pharmakol.*, 1913, lxxi, 364.
37. Vaquez and Lutembacher, *Bull. Acad. méd.*, 1917, lxxvii, 405.
38. Macht, D. I., and Colson, H., *J. Pharmacol. and Exp. Therap.*, 1917, ix, 343.
39. Richards, A. N., and Wood, W. G., *J. Pharmacol. and Exp. Therap.*, 1914–15, vi, 283.
40. Clark, A. J., *Brit. Med. J.*, 1912, ii, 687.

41. Voegtlin, C., and Macht, D. I., *J. Pharmacol. and Exp. Therap.*, 1913–14, v, 77.

42. Fraser, T. R., *Tr. Roy. Soc. Edinburgh*, 1891, xxxvi, 343.

43. Schmiedeberg, O., *Arch. exp. Path. u. Pharmakol.*, 1910, lxii, 305.

EXPLANATION OF PLATES.

Plate 23.

Fig. 1, *a*. Cat 12 d. This electrocardiogram was taken at 2.40 p.m. before strophanthin injections.

b. This curve was taken at 4.44 when 36 per cent of the minimum lethal dose had been given. It shows ventricular extrasystolic tachycardia, the typical toxic effect.

c. Curve taken at 5.43. 10 per cent in addition had been given at 5.42 (total of 46 per cent of the minimum lethal dose). It shows typical ventricular fibrillation.

Fig. 2, *a*. Cat 6 b. Curve taken at 3.35 p.m. before strophanthin was injected. It shows normal heart mechanism.

b. Curve taken at 4.13 after 35 per cent of the minimum lethal dose had been given. It shows a toxic effect in which the ventricular complex is continually changing. There are alterations also in the P–R relation.

Plate 24.

Fig. 3, *a*. Cat 12 a. Curve taken at 4.57 p.m. before strophanthin injection. The first five contractions are nodal beats.

b. Curve taken at 5.05 after 20 per cent of the minimum lethal dose of strophanthin had been given. It shows a nodal rhythm; the auricular waves are lost in the ventricular complexes.

c. Curve taken at 5.07 after 28 per cent of the minimum lethal dose. It shows that the heart has returned to the normal mechanism.

Plate 25.

Fig. 4, *a*. Cat 7 b. Curve taken at 12.15 p.m., before strophanthin was given. It shows normal rhythm.

b. Curve taken at 1.06 p.m., after 52 per cent of the minimum lethal dose had been given. It shows frequently changing ventricular complexes.

c. Curve taken at 1.15. No additional strophanthin. The curve shows ventricular fibrillation in the first portion and then a standstill of the heart.

d. Curve taken at 1.30 showing the gradual onset of a ventricular extrasystolic tachycardia.

e. Curve taken at 1.37 showing sequential contraction of auricles and ventricles but with delayed conduction; $P-R = 0.16$ second.

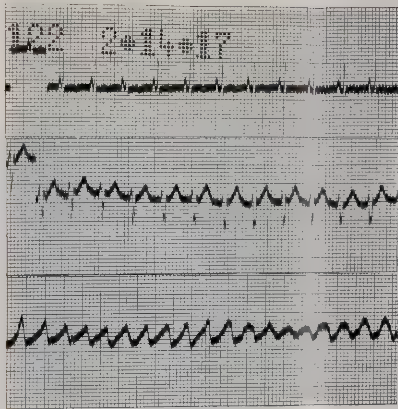

FIG. 1.

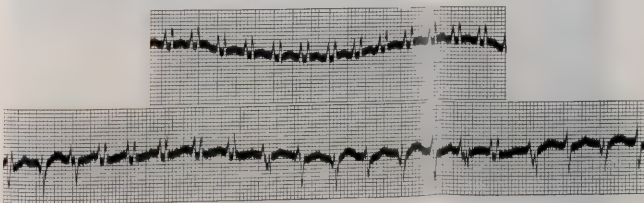

FIG. 2.

(Levine: Action of strophanthin on cat's heart.)

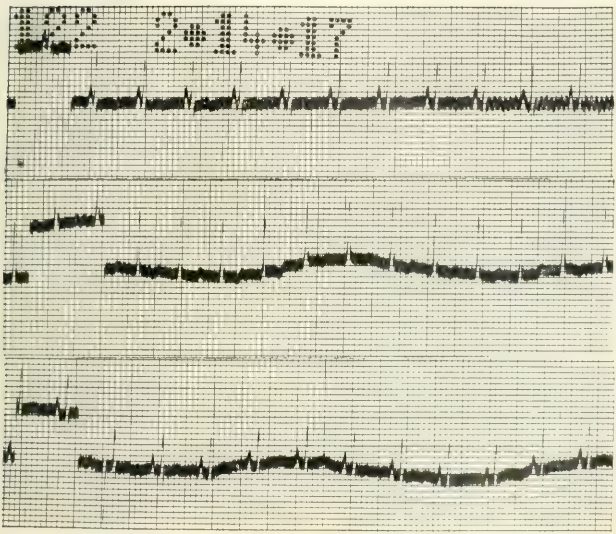

FIG. 3.

(Levine: Action of strophanthin on cat's heart.)

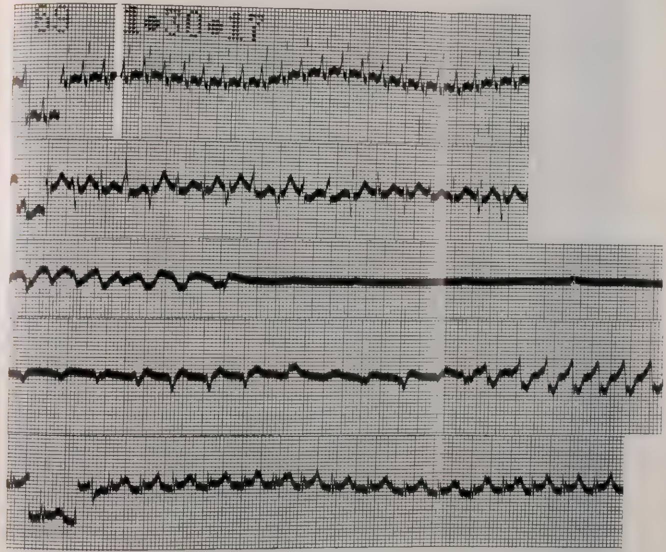

FIG. 4.

(Levine: Action of strophanthin on cat's heart.)

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STUDIES OF ACIDOSIS.

XIII. A Method for Titrating the Bicarbonate Content of the Plasma.

By DONALD D. VAN SLYKE, EDGAR STILLMAN, and GLENN E. CULLEN.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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For determination of carbonates and bicarbonates in the absence of proteins and other buffers methods of two general types are available: (1) measurement of the carbon dioxide evolved when the carbonates are decomposed with acid, and (2) titration of the basic part of the carbonate molecule, either by adding excess acid, boiling off the CO_2, and titrating back, or by using an indicator which, like methyl orange, is not sensitive to the weak acidity of carbonic acid. Hitherto only methods of the first type, based on carbon dioxide determination, have proven accurate and practicable for blood analysis.

In order to titrate accurately the bicarbonate of the blood plasma, it is both theoretically and actually necessary, as a net result of the operation, to transform the bicarbonate into the salt of whatever acid is used in the titration, without altering the normal hydrogen ion concentration of the plasma. If the hydrogen ion concentration is altered, the proteins of the plasma bind as a result either acid or alkali in amounts different from those which they bind *in vivo*. Consequently, under such conditions the proteins act either to increase the acid added in titration to an amount more than equivalent to the bicarbonate, or to depress it below the bicarbonate equivalent.

So far as we are aware, no method for titration of the blood plasma with indicators has yet been proposed which meets the following three requirements, all of which are necessary in order to permit interpretation of the results in terms of plasma bicarbonate: (1) The use of the hydrogen ion concentration of normal blood as the end-

point; (2) Removal of the carbon dioxide set free by the added acid; (3) Use of an indicator not affected by the plasma proteins.

It appeared probable that if these requirements could be met, a simple titration of the plasma bicarbonate might be possible, and recent investigations have provided the theoretically necessary data. The work of Lundsgaard (1912), who first demonstrated the constancy of the hydrogen ion concentration of the blood, of Hasselbalch (1911, 1913, 1915), and of a number of subsequent investigators (Michaelis, 1914; Peabody, 1914; Milroy, 1914; Parsons, 1917; Sonne and Jarløv, 1918), has shown that the pH of human blood under nearly all conditions, normal and pathological, even in acidosis, is approximately constant at 7.4. McClendon has shown that when excess acid is added to the blood plasma, the CO_2 driven off by a stream of hydrogen, and alkali added until the normal pH, as determined by the gas chain, is restored, the acid consumption is about what would be expected from the average bicarbonate content established by Van Slyke, Stillman, and Cullen (1917). An indicator capable of showing pH 7.4 accurately, in the presence of the plasma proteins, has been found in neutral red by Miss Annie Homer (1917). The elimination of carbon dioxide is merely a matter of efficient aeration. Consequently the difficulties to the titration appear to have become theoretically surmountable, and we have found in fact that the simple titration technique described below gives results for plasma bicarbonate identical with those yielded by determining the carbon dioxide.

Description of Method.

In drawing and centrifugating the blood the precautions outlined by Van Slyke and Cullen (1917), for preventing loss or accumulation of carbon dioxide and consequent change in the distribution of bicarbonate between corpuscles and plasma, are to be observed. Oxalate plasma is used. Up to the beginning of the analysis, the blood and plasma are handled exactly as described for the carbon dioxide method.

For the analysis 2 cc. of plasma are pipetted into a round-bottomed flask of 150 to 200 cc. capacity, and 5 cc. of 0.02 N hydrochloric acid are added (this is about 2 cc. of 0.02 N acid in excess of the bicarbonate

normally present). In order to remove the carbon dioxide set free by the acid, the flask is shaken vigorously with a rotary motion, so that the solution is whirled in a thin layer about the inner wall. 1 minute of this treatment is sufficient to remove the carbonic acid so completely that not enough is left to affect the results measurably. The solution is now poured as completely as possible into a 50 cc. Erlenmeyer flask and the walls of the larger flask are rinsed with 20 cc. of water. The water is measured within 1 cc. in a cylinder, and approximately a third is used for each of three washings.

When the solution, measuring about 26 cc., has been transferred to the 50 cc. Erlenmeyer flask, 0.3 cc. of a 0.1 per cent solution of neutral red (dissolved in 50 per cent alcohol) is added. 0.02 N *carbonate-free* NaOH is then run in from a burette (preferably but not necessarily a "micro-burette") until the color of the solution matches that of 29 cc. of a standard phosphate solution, of pH 7.4, contained in a similar 50 cc. flask.[1]

In place of neutral red, 0.3 cc. of a 0.04 per cent solution of phenolsulfonephthalein may be used as indicator, and gives an end-

[1] The standard solutions of pH 7.2 and 7.4 may be made as follows (Clark and Lubs, 1917), M/20 solutions being obtained. The M/5 KH_2PO_4 contains 27.23 gm. of KH_2PO_4 per liter.

pH	M/5 KH_2PO_4	N/5 NaOH	
	cc.	*cc.*	
7.2	50	35.0	Dilute to 200 cc.
7.4	50	39.5	" " 200 "

They may also be made by Sörensen's method (Sörensen, 1912) from KH_2PO_4 and Na_2HPO_4 as follows, the phosphate concentration being M/15. Both salts prepared especially for this purpose may now be obtained from Merck and Co.

pH	Na_2HPO_4	KH_2PO_4	
	gm.	*gm.*	
7.2	6.89	2.47	Dilute to 1 liter.
7.4	7.72	1.67	" " 1 "

If crystalline $Na_2HPO_4.2H_2O$ is used instead of the anhydrous Na_2HPO_4, the amounts are increased to 8.66 and 9.67 gm. for the solutions of pH 7.2 and 7.4 respectively.

The plasma solution becomes somewhat turbid, but the turbidity partially clears up as the end-point is approached, and the latter can be determined within one drop of the 0.02 N NaOH.

point slightly more easy to distinguish than that of neutral red. When phenolsulfonephthalein is used, however, the standard solution must be of pH 7.2 instead of 7.4. The reason for this is that, as pointed out by Miss Homer (1917), when phenolsulfonephthalein is added to a solution containing serum proteins, the pH is considerably higher than that estimated by colorimetric comparison with standard phosphate solutions. The indicator suffers from a protein error. Under the conditions of the analysis here described, however, the error is quite constant at 0.2 to 0.3 pH; *i.e.*, if the color matches that of a standard solution of pH 7.4, the actual pH in the solution under examination is about 7.7 (see Table II). If a standard solution of pH 7.2 is used, however, the titration runs to the same point obtained with neutral red, and, as stated above, the end-point is, to most eyes, somewhat sharper than with neutral red.

For the titrations we have used a 3 cc. micro-burette of the model devised for use with the blood sugar method of Bang. It was provided with an automatic filling device and divided into 0.02 cc. divisions which were over 1 mm. apart, so that readings accurate to within 0.01 cc. could be made easily. The tip was drawn out to a fine point, so that the drops were small, measuring only 0.03 cc. each. Such a burette is extremely desirable, but not absolutely necessary. Even an ordinary 50 cc. burette divided into 0.1 cc. divisions may be used, an error of 0.1 cc. altering the result by only one-thirtieth of the normal, since the bicarbonate in 2 cc. of plasma normally neutralizes about 3 cc. of 0.02 N acid. The accuracy of the method, however, justifies the use of a finer burette, for our results seldom varied from the calculated values, or from agreement with duplicates, by more than 0.05 cc. and were usually within one drop, or 0.03 cc.

The End-Point.—With both indicators, a peculiar phenomenon occurs as the end-point is approached. Each drop appears to change the color past the end-point, but within a few seconds the color shifts back, and it is seen that at least another drop is needed before the genuine end-point is reached. Consequently, the final color comparison should not be made until at least 30 seconds after the last drop of 0.02 N NaOH has been added. Because of this behavior, as well as the character of the color change, it is well, par-

ticularly with neutral red, to overrun the end-point by a drop, rather than stop short of it when in doubt.

Calculation of Results.—The number of cc. of 0.02 N NaOH used in the titration is subtracted from the number required to neutralize to the same indicator 5 cc. of the 0.02 N HCl used. This number is, of course, approximately 5, but it usually varies slightly from that because of difference in the factors of acid and alkali, and because of the calibration error of the 5 cc. pipette used for measuring the acid. Consequently the maximum accuracy is obtained by performing a preliminary titration on 5 cc. of the acid plus 20 cc. of the distilled water, using the same pipette, indicator, and end-point as in the plasma titration.

The following is a typical calculation on a normal human plasma.

0.02 N NaOH = HCl added	5.09 cc.
0.02 N NaOH taken in titration	2.03 "
0.02 M $NaHCO_3$ in 2 cc. plasma or 0.01 M $NaHCO_3$ in 1 cc. plasma	3.06 "

$3.06 \div 100 = 0.0306$ = molecular concentration of $NaHCO_3$ in plasma.

$3.06 \times 22.4 = 68.5$ volume per cent CO_2 bound as bicarbonate in the plasma.

Since the titration result represents cc. of 0.01 M $NaHCO_3$ per cc. of plasma, it is transformed into terms of molecular concentration of $NaHCO_3$ in the plasma merely by dividing by 100.

For the sake of comparison with results of bicarbonate determination by the CO_2 method (Van Slyke and Cullen), the molecular concentration is multiplied by 2,240, in order to give results in terms of cc. of CO_2 per 100 cc. of plasma. According to the gas laws the amount of CO_2 contained in a M carbonate solution is 22,400 cc. per liter (measured as CO_2 gas at 0°, 760 mm.) or 2,240 cc. of gas per 100 cc. of solution. Hence multiplying the bicarbonate molecular concentration by 2,240, or multiplying the cc. of 0.02 N acid used in the titration by 22.4, gives the volume per cent of bicarbonate CO_2 in the plasma.

Inversely, of course, dividing the volume per cent of CO_2, as determined by Van Slyke and Cullen, by 2,240 transforms the CO_2 figures into terms of molecular concentration.

The Standard 0.02 N *Sodium Hydroxide.*—The 0.02 N NaOH as a basis for the determination must, in order to maintain its value,

be protected from contact with atmospheric carbon dioxide and from glass. Even standing over night in a burette of soft glass is likely to result in the solution of enough alkali to raise the titration value of the standard solution. The standard alkali should be kept in paraffined bottles, and the burette filled with fresh solution each day that it is used.

In order to obtain a carbonate-free alkali solution, we use the well known expedient of first dissolving the NaOH in an equal weight of water. Sodium carbonate is insoluble in such a concentrated alkali solution and settles to the bottom. 5.5 cc. of the clear supernatant solution diluted to 5 liters yields an approximately 0.02 N solution which is standardized by titration with neutral red against 0.02 N HCl. In performing the titration it is preferable to run the acid into the alkali, thus titrating from the yellow alkaline color to the acid red. The color change in this direction occurs without the time lag observed when alkali is added to acid.

EXPERIMENTAL.

Determinations on Known Carbonate Solutions.—Solutions of Merck's C.P. Na_2CO_3 were made in concentrations ranging from the carbonate content of normal plasma downwards, and titrated as described for plasma. The results are given in Table I.

Accuracy of End-Point in Plasma Titrations.—Table II shows the results of a number of titrations. After the titrations were finished the pH values of the solution were checked by means of the gas chain. It is seen that the desired value of pH 7.4 was closely approximated as the end-point in each titration, when neutral red was used as indicator with a standard of pH 7.4 for comparison, or when phenolsulfonephthalein was used as indicator with a standard of pH 7.2 for comparison. With the latter indicator, however, and a color standard of pH 7.4, the titration due to the protein error pointed out by Miss Homer (1917), went to a pH of 7.7. Even this change altered the results by only about 0.001 M, and when a color standard of pH 7.2 was used with phenolsulfonephthalein, the error was completely corrected.

Time Required for Removal of Carbon Dioxide by Aeration.—A series of determinations was performed with portions of the same

TABLE I.

Na_2CO_3 per liter by weight.*	Concentration of carbonate by methyl orange titration.		Concentration of carbonate by neutral red titration with technique used for blood plasma.	
	0.02 N HCl = 20 cc. carbonate solution.	Concentration calculated as $NaHCO_3$.	0.02 N HCl = 2 cc. solution.	Concentration calculated as $NaHCO_3$.
gm.	*cc.*	*molecular*	*cc.*	*molecular*
1.590	29.1	0.0291	2.90	0.0290
1.090	19.7	0.0197	1.95	0.0195
0.727	13.1	0.0131	1.29	0.0129
0.363	6.57	0.0066	0.67	0.0067

* The carbonate was not dried, hence the actual concentration indicated by the methyl orange titration is only 97 to 98 per cent that indicated by the weight of carbonate taken.

TABLE II.

Serum.	0.02 N NaOH = HCl taken.	0.02 N NaOH used in back titration.	0.02 N NaOH = $NaHCO_3$ in 2 cc. plasma.	Concentration of $NaHCO_3$.	Indicator used.	pH of standard phosphate solution used for end-point comparison.	pH of plasma solution at end of titration.
	cc.	*cc.*	*cc.*	*molecular*			
Horse 1.	5.13	2.41	2.72	0.0272	Neutral red.	7.4	7.36
	5.13	2.44	2.69	0.0269	" "	7.4	7.41
	5.12	2.56	2.56	0.0256	Phenolsulfonephthalein.	7.4	7.72
	5.12	2.53	2.59	0.0259	"	7.4	7.71
	5.12	2.43	2.69	0.0269	"	7.2	7.45
Horse 2.	5.35	2.60	2.75	0.0275	Neutral red.	7.4	7.43
	5.35	2.63	2.72	0.0272	Phenolsulfonephthalein.	7.2	7.45

TABLE III.

Duration of aeration.	Bicarbonate concentration found by titration.
min.	*molecular*
0	0.0117
0.25	0.0220
0.5	0.0255
1.0	0.0260
2.0	0.0260
3.0	0.0260

horse serum, exactly as outlined above under "Description of Method," except that the aeration period was varied. The results in Table III show that an aeration period of 1 minute is sufficient, but that 30 seconds is probably not quite enough. Incidentally the result of the titration performed without aeration shows the impossibility of a correct titration without removal of carbon dioxide.

Parallel Determinations of Plasma Bicarbonate by Titration and by Carbon Dioxide Capacity.—The results in Table IV were obtained on the plasmas of blood samples drawn from the ear veins of rabbits and the arm veins of men. The carbon dioxide capacities were determined by one of us, while the titrations were being performed by another.

TABLE IV.

Plasma.	CO_2 bound as bicarbonate by 100 cc. of plasma.	Bicarbonate concentration calculated from $CO_2\left(=\frac{CO_2}{2,240}\right)$.	Bicarbonate concentration determined by titration.
	cc.	*molecular*	*molecular*
Normal Rabbit 61	56.7	0.0253	0.0270
" " 62	66.9	0.0298	0.0305
" " 63	46.2	0.0206	0.0199
Diabetic. No acidosis	58.2	0.0259	0.0240
Normal man	61.6	0.0275	0.0265

Parallel Determinations by Titration and Carbon Dioxide Capacity Methods on Normal Human Plasma plus Known Amounts of Acetic Acid.—Acetic acid has practically the same dissociation constant (1.8×10^{-5}) as β-hydroxylbutyric (2.0×10^{-5}), and the effect on acid-base balance produced by adding it to plasma is therefore practically identical with the effect of β-hydroxylbutyric acid.

The blood was drawn from one of the authors. 5 cc. portions were treated with water, or water plus 0.1 N acetic acid, making the total volume up to 6.5 cc. Portions were then taken for titration and for carbon dioxide capacity determination (Van Slyke and Cullen method), as described in the preceding paragraph. Table V gives the results. The CO_2 results are reduced to terms of molecular concentration by dividing the volume per cent CO_2 by 2,240, the theoretical gas content, as described on page 573. The results are plotted in Fig. 1.

Discussion of the Titration and Carbon Dioxide Capacity Methods for Determination of Plasma Bicarbonate.

It is evident from Fig. 1 that the titration gives theoretically correct values over the entire range of bicarbonate concentrations from normal to zero. The CO_2 determinations agree both with the titration results and with the calculated values for a range of the latter from normal down to somewhat below 0.01 M bicarbonate (22.4 per cent CO_2 capacity). When the bicarbonate falls as low as 0.003 M, however, (6.7 per cent CO_2, or about one-tenth the normal), the CO_2 method gives higher bicarbonate than is actually present.

TABLE V.

Plasma.	0.1 N acetic acid	Water.	CO_2 bound as bicarbonate per 100 cc. of treated plasma.	Concentration of bicarbonate.		Fall in concentration.		Concentration of acetic acid added.
				By CO_2 capacity.	By titration.	By CO_2 capacity.	By titration.	
cc.	*cc.*	*cc.*	*cc.*	*molecular*	*molecular*	*molecular*	*molecular*	*molecular*
5	0	1.5	58.9	0.0267	0.0264	0	0	0
5	0.478	1.0	41.6	0.0186	0.0192	0.0081	0.0072	0.0073
5	1.036	0.5	24.2	0.0108	0.0106	0.0155	0.0158	0.0159
5	1.514	0.0	12.8	0.0057	0.0028	0.0205	0.0236	0.0233
5	1.036 of 0.2 N	0.5	0.0	0.0000	−0.0050	—	0.0314	0.0319

This fact was demonstrated and discussed in the original paper on the carbon dioxide capacity method (Van Slyke and Cullen, pp. 327, 328) in the following words:

"Until acid equivalent to about half the plasma bicarbonate has been added the fall in bicarbonate approximately equals in molecular equivalents the amount of acid added. As the amount of acid becomes greater, however, the drop in plasma bicarbonate begins to fall short of the added acid. This is due to the fact that the H_2CO_3 concentration is kept constant, instead of being reduced in proportion to the bicarbonate. The condition is similar to that of the blood in uncompensated acidosis. The $\frac{H_2CO_3}{NaHCO_3}$ ratio, and consequently the C_H is increased. As a result the other plasma buffers (chiefly proteins) bind a measurably greater amount of acid than they could at normal C_H, and the acid so bound is prevented from decomposing bicarbonate.

"The effect on the routine plasma determination is that the bicarbonate determined by our [CO_2] technique denotes a fall in the more severe stages of acidosis which is not quite so great as the actual drop in bicarbonate *in vivo*. The relationship between added acid and decrease in bicarbonate, however, is made so constant by saturating the plasma at a definite carbon dioxide tension that the lack of absolute numerical proportionality in the lower ranges is no practical detriment to the interpretation of results."

In some types of experimental work the theoretical perfection of the titration will doubtless give it an advantage, but for ordinary

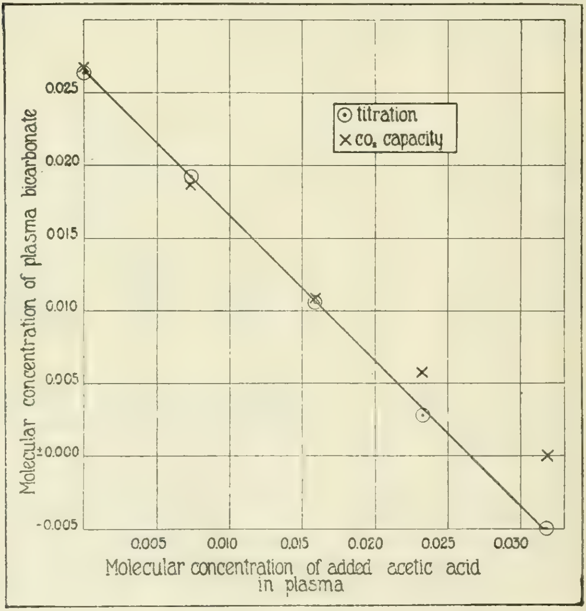

FIG. 1. Effect of addition of acetic acid on bicarbonate concentration of normal human plasma. The straight line represents the bicarbonate concentration calculated on the assumption that the fall in bicarbonate is equivalent to the amount of acid added to the plasma. The results of titrations are represented by ⊙, the results of CO_2 determinations by +.

purposes, especially for clinical diagnosis, the choice between the two methods appears entirely one of convenience. The carbon dioxide capacity has the advantage that the extraction and measurement of the gas require slightly less time than even the few minutes necessary for a titration, and require no accurate standard solutions; while the titration has the advantage that it requires no special apparatus. Within the bicarbonate range ordinarily encountered even in acidosis (Stillman, Van Slyke, Cullen, and Fitz, 1917), the results of either method apparently may be interpreted in terms of the other, so closely do the two agree, and each method is capable of accuracy to within 1 or 2 per cent of the amount of be carbonate normally present.

CONCLUSION.

The bicarbonate content of serum or oxalate plasma is determined by adding an excess of standard acid (5 cc. of 0.02 N HCl to 2 cc. of plasma), removing the carbon dioxide by rotating the solution for 1 minute about the wall of a flask, and titrating back with 0.02 N NaOH to the original hydrogen ion concentration of blood (pH 7.4) with neutral red as indicator.

The results agree closely with those of the carbon dioxide capacity method over the range of bicarbonate concentrations (0.03 to 0.01 M) ordinarily encountered in man, even in severe acidosis. Below this range the titration continues to give accurate results, while the CO_2 capacity method gives, for reasons discussed in the original paper on the CO_2 method, somewhat higher values. For clinical and most experimental purposes, however, it appears that the two methods give so nearly identical results that they may be used interchangeably.

Micro-Titration.—When it is desirable to employ only small amounts of plasma, 0.400 cc. may be transferred to a test-tube of about 20 mm. diameter, and 1.000 cc. of 0.02 N HCl added. The tube is laid in a nearly horizontal position, so that the layer of liquid extends for about 10 cm. along the lower side. The tube is then rotated or rolled back and forth vigorously, but not so roughly as to cause foaming, for not less than 1.5 minutes, in order to cause the carbon dioxide to escape. Three drops of indicator are then added, and the solution is titrated in the test-tube with 0.004 N NaOH to a pH of 7.4, using as a color standard a like volume of phosphate solution in a similar tube.

The 0.004 N NaOH is so rapidly altered by contact with either glass or atmospheric carbon dioxide that it is advisable to make it fresh for each series of analyses by diluting 10 cc. of 0.1 N NaOH to 250 cc. with distilled water that has been freed of CO_2 either by boiling or by shaking in an evacuated flask. The control titration of the 0.004 N NaOH against the 1 cc. of 0.02 N HCl should be performed immediately before the plasma titration.

The calculation is the same as in the larger titration. The number of cc. of 0.004 N NaOH used is subtracted from the number of cc. (approximately 5) required to neutralize the HCl in the control titration. The difference divided by 100 represents the molecular concentration of bicarbonate in the plasma, while the difference multiplied by 22.4 indicates the volume per cent of bicarbonate CO_2.

With care in the calibration of pipettes, and especially in the control of the 0.004 N NaOH, results nearly and perhaps quite as accurate as in the larger titration appear attainable.

BIBLIOGRAPHY.

Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 26.

Hasselbalch, K. A., *Biochem Z.*, 1911, xxx, 317; *ibid.*, 1913, xlix, 451. Hasselbalch, K. A., and Gammeltoft, S. A., *ibid.*, 1915, lxviii, 206.

Homer, A., *Biochem. J.*, 1917, xi, 283.

Lundsgaard, C., *Biochem. Z.*, 1912, xli, 247.

McClendon, J. F., *J. Biol. Chem.*, 1918, xxxiii, 19.

Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.

Milroy, T. H., *Quart. J. Exp. Physiol.*, 1914–15, viii, 141.

Parsons, T. R., *J. Physiol.*, 1917, li, 440.

Peabody, F. W., *Arch. Int. Med.*, 1914, xiv, 236.

Sörensen, S. P. L., *Ergebn. Physiol.*, 1912, xii, 393.

Sonne, C., and Jarløv, J., *Hospitalstid.*, 1917, lx, 1247; abstracted in *Chem. Abst.*, 1918, xii, 1893.

Stillman, E., Van Slyke, D. D., Cullen, G. E., and Fitz, R., *J. Biol. Chem.*, 1917, xxx, 405.

Van Slyke, D. D., Stillman, E., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 401.

Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 317.

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FIRST ACCOUNT OF A THERMOTROPISM IN ANOPHELES PUNCTIPENNIS, WITH BIONOMIC OBSERVATIONS.

By WERNER MARCHAND.

(From the Department of Animal Pathology, The Rockefeller Institute for Medical Research, Princeton, N. J.)

In the fall of 1915, while taking part in a survey of the breeding-places of Anopheline mosquitoes in the neighborhood of Princeton, in coöperation with the local Mosquito Extermination Commission, the writer made certain observations on the mosquitoes encountered. This account is restricted to a few facts which appear to be new or serve to clear up some doubtful point in the life history of the mosquitoes.[1]

I. Bionomics of the Larvæ.

In the Princeton region, only two species of Anopheles have been recorded, these being *A. quadrimaculatus* and *A. punctipennis*. The latter species is by far the more common, but, since King's experiments (1916),[2] it cannot be regarded as entirely harmless. It is doubtful, however, whether this species, which has been found to occur as far north as Boston, Mass. (Th. Smith),[3] is also in the northern states a regular carrier of malaria.

The larvæ of *A. punctipennis* were kept captive in large numbers and lived best in a flat dish which was left uncovered in order to give free access to the air. In a dish about eight inches in diameter, filled to a depth of about one and one-half inches with water from a pond, more than two hundred Anopheles larvæ, mostly

[1] The writer wishes, on this occasion, to express his thanks for the kind helpfulness through which his work was facilitated by Professor E. G. Conklin and Professor Ulric Dahlgren of Princeton University. Dr. Conklin also had the kindness to revise the English of the MS.

[2] King, W. V. Experiments on the development of malaria parasites in three American species of Anopheles. Jour. Exp. Med., Vol. xxiii, pp. 703–716, 1916.

[3] Smith, Theobald. Notes on the occurrence of *Anopheles punctipennis* and *Anopheles quadrimaculatus* in the Boston suburbs. Jour. Bost. Society of Medical Sciences, Vol. V, pp. 321–324, 1901.

collected in a half-grown stage, many of them in very young stages, developed into pupæ. The pupæ when formed were taken out with a pipette and transferred to another jar with provisions for the adults to hatch.

In order to rear Anopheles larvæ successfully, it is necessary to feed them, and this fact has not been sufficiently emphasized. It was found that certain unicellular surface algæ, the species of which could not be determined, form a very satisfactory food. These algæ developed freely in one of the glass-covered aquaria in the Vivarium of Princeton University. In this aquarium they covered the whole surface of the water in a continuous green sheet, which was wrinkled and folded in places through the excessive development of the algæ. In order to transfer these to the culture dishes it was sufficient to dip the finger into this stratum and then dip it into the Anopheles-basin; here the algæ, upon reaching the clear surface of the water, would immediately spread out over the whole surface in the form of a thin, opaque, green layer. The Anopheles larvæ would at once start feeding upon these minute algæ, a clear area soon forming around the head and mouthparts of each larva. Before evening, the larvæ had cleared the whole surface of algæ. The procedure was repeated at night in order to provide food for the larvæ to last until the next morning, when again the algæ had been completely devoured. In this way they were fed regularly twice daily. Under ordinary conditions, when Spirogyra or other filamentous algæ are given as food (C. A. Smith),[1] many larvæ usually die, because, as a matter of fact, their natural way of feeding is at the surface. Howard, Dyar, and Knab (1912)[2] suggest that to provide food the jars in which Anopheles larvæ are grown, should be kept uncovered, "in order that the dust from the air may settle continuously upon the water." Undoubtedly, the Anopheles larvæ may feed on surface bacteria or protozoa, but in absence of green plants, the contamination of the water often causes death. When surface algæ are given, as in the case here recorded,

[1] Smith, Cora A. The Development of *Anopheles punctipennis* Say. Psyche, Vol. XXI, p. 1, 1914.

[2] Howard, Dyar, and Knab. The mosquitoes of North and Central America and the West Indies, Vol. I, 1912.

practically no larvæ are lost, because these algæ produce oxygen in abundance, and the water does not have to be renewed at all. The quantity of algæ devoured by the larvæ was quite considerable. Lack of attention to this detail may perhaps explain the frequent failures in rearing Anopheles larvæ.[1] On the other hand, the use of surface algæ as food may serve to determine with exactness the quantity of food consumed by a single larva, since the algæ, under carefully chosen conditions, cover the surface quite evenly in a layer of measurable thickness, and therefore the quantity of algæ present on a surface of given dimensions and consumed in a given time may be estimated or calculated, and, divided by the number of larvæ feeding on this surface, would give the quantity consumed by a single larva. However, as the season was advanced, and the larvæ were transforming into pupæ, this experiment was not carried out.

The larvæ showed in a remarkable degree the characteristic instinct spoken of by Zetek,[2] to drop to the bottom when a shadow passed over their heads. When the writer came near them, in the morning, after they had been completely undisturbed for many hours, the phenomenon was particularly striking. The larvæ would drop almost simultaneously and then would remain at the bottom for several minutes.

In this connection, it may be noted that Graham has stated that in the Sudan, microscopic fresh-water algæ form the principal food of Anopheles, a fact not unimportant for their control, since it may be that the mosquitoes may be kept in check by methods aiming at a destruction of the algæ.[3]

[1] Graham, W. M. (A study of mosquito larvæ, Jour. Ent. Research, Vol. I, 1910) has stated correctly that failure to rear the larvæ is not to be wondered at when it is recognized that mosquito larvæ require a constant supply of special food, consisting usually of living fresh-water algæ. In the absence of algæ the larvæ become cannibalistic and destroy one another.

[2] Zetek, James. Behavior of *Anopheles albimanus* Wiedemann and *tarsimaculatus* Goeldi. Ann. Ent. Soc. of America, Vol. VIII, p. 221 ff., 1915.

[3] Graham, loc. cit.; these algæ were not surface algæ but were suspended in the water; as stated, however, the Anopheles larva is mainly a surface feeder.

II. Bionomics of the Adult Stage.

The resting position of Anopheles has often been used as a characteristic to distinguish the malarial mosquito from other species, the Anopheles holding the body, as a rule, at a certain angle to the surface on which they are resting. This angle is, in *A. punctipennis*, usually about 45°; Nuttall and Shipley's illustration as reproduced by J. B. Smith (Mosquito-control exhibit, N. J. State Museum),[1] in which it is represented to form a right angle with the surface, is an extreme case and not quite typical. It seems, however, that mosquitoes which rest for many hours in the same place, assume a more oblique position than do these which have just alighted. These characteristics apply only to *Anopheles punctipennis*, not to *A. quadrimaculatus*. Hibernating females of the latter species were observed beyond doubt in a resting position in which the body was held about parallel to the resting surface, as illustrated in the accompanying drawing from nature (Fig. 1). Hence, they can be easily mistaken for Culex if only the resting position is taken as a criterion.[2]

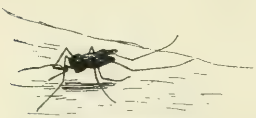

FIG. 1. Resting position of hibernating female of *Anopheles quadrimaculatus*.
Sketch-drawing from life.

Concerning the biting position of Anopheles, Nuttall and Shipley's illustration (J. B. Smith, N. J. State Museum exhibit) is not entirely correct (Washburn),[3] and H. P. Johnson[4] is in error in assuming that the mosquito must necessarily bite with proboscis inserted at a right angle. As a matter of fact, *A. punctipennis* will insert the proboscis usually at an oblique angle, the mosquito biting in a posi-

[1] Nuttall and Shipley. The structure and biology of *Anopheles maculipennis*. Jour. of Hygiene, 1901.

[2] Howard (loc. cit. p. 205) has recorded the same fact for *A. quadrimaculatus* hibernating in barns in southern Idaho.

[3] Washburn, F. L. Economic Entomology at the Worlds' Fair. Science, N. S., Vol. XX, No. 518, 1904, and "The biting position of Anopheles." Science, N. S., Vol. XXI, p. 228, 1905.

[4] Johnson, H. P., quoted by Smith, J. B. How does Anopheles bite? Science, N. S., Vol. XXI, pp. 71–72, 1905.

tion much like the resting position, and the line of the proboscis forming the continuation of the longitudinal axis of the body. This is evidently of advantage for the sucking mechanism.

III. The Biting Instinct Due to a Thermotropic Reaction.

Observations were also made on the biting instinct, which as it seems, is determined mainly by thermotropism. Patton and Cragg (1913) have reported[1] that Howlett observed that females of *Stegomyia scutellaris* were attracted by the hot air radiating from a test tube filled with hot water. On the other hand, shed blood and sweat did not attract the ♀♀ of this species and *Culex fatigans*

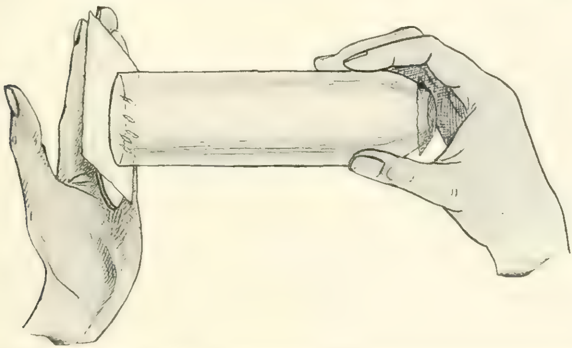

FIG. 2. Arrangement used in demonstrating the thermotropism of mosquitoes.

any more than water. Howlett's experiments were not known to me at the time when these observations were made, and since they were obtained independently of other observers, and the phenomenon was not known to apply to Anopheles, I may briefly report on them here, especially as data on thermotropism in insects are very scanty. The Anopheles which were kept in lamp chimneys for other purposes, were fed on apple jelly which was spread out on a

[1] Patton and Cragg. Textbook of Medical Entomology, London, Madras and Calcutta, 1913.

glass plate. In order to prevent the mosquitoes soiling their legs and wings, the jelly was covered with filter paper. In the intention of providing a food as natural as possible, I heated the apple jelly on the glass plate, assuming that it would then be taken more readily. This was in fact the case. The mosquitoes came quickly to the filter paper and would bite through it as if it were human skin. The question suggested itself whether they were attracted by the odor of the jelly or, possibly, by the heat radiating from it. Being curious whether the mosquitoes would be attracted also by heat alone, I substituted for the glass plate which was covered with jelly, a clean one which was heated to a degree fairly above human body temperature but, of course, not excessive, and covered with filter paper in the same way as before, in order to provide a foothold for the insects. The mosquitoes were attracted under these conditions in the same way as if food had been present, each one attacking the surface of the filter paper which covered the glass plate, and all bending their proboscis in repeated efforts to pierce the surface. The number of mosquitoes used was about five or six at a time. The arrangement was that given in the diagram (Fig. 2). If several mosquitoes are used in biting experiments, there will usually be some individuals which will show no inclination to bite, but the percentage of individuals not attracted by the heated glass plate, was about the same, and not greater than in the biting experiments. The males showed the same tropism as the females but much less strongly. As soon as the glass plate had cooled off, the mosquitoes became indifferent. However, the experiment could be repeated with the same mosquitoes as also with different sets and always with the same results.

These observations, taken together with those made by Howlett, indicate that this thermotropic reaction is a very important factor to be considered in the analysis of the bloodsucking instinct.

I have, since, tried to confirm these facts by observations on other species of mosquitoes, but so far have used only the hibernating females of *Aëdes sylvestris*. These, however, did not show even a trace of the thermotropic reaction observed in *A. punctipennis*. On the other hand they also consistently refused to bite, though various food other than blood was readily accepted. Fe-

males of Anopheles are known to bite occasionally in winter, and therefore, usually hibernate in stables where blood can be obtained (Grassi and others; see Howard, Dyar, and Knab)[1] while *Aëdes* which hibernates in cellars, seems not to bite at all during the winter even if brought into a heated room. The absence of thermotropism would, therefore, in this case, be only an adaptation to the conditions of hibernation, during which no blood food is taken, and it is perfectly possible, that *Aëdes sylvestris* will be found thermotropic during the "biting season" unless, in this genus, other tropisms nvolved.

[1] Loc. cit., pp. 206–209.

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A CHARACTERISTIC LOCALIZATION OF BACILLUS ABORTUS IN THE BOVINE FETAL MEMBRANES.

BY THEOBALD SMITH, M.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

PLATES 20 TO 22.

(Received for publication, February 21, 1919.)

While making a study of the diseased membranes in cases of infectious abortion of cattle, the writer came upon a peculiar and characteristic habitat of *Bacillus abortus* Bang, in the epithelial covering of the chorion.

This layer of cells which faces the epithelial covering of the uterine mucosa and is in intimate contact with it covers the intercotyledonous areas of the chorion and is continuous with the epithelium of the villosities of the cotyledons which dip into the depressions of the maternal caruncles. The general character of the cells may be seen in Fig. 4. The cells vary somewhat in height. The vesicular nucleus is round or oval in outline and the chromatin appears as minute 1 μ spheres against the nuclear membrane. The free border of the cytoplasm appears frequently in the form of blunt finger-like or conical projections, giving the surface a fimbriated appearance. In the specific infectious disease of the fetal membranes these cells, either individually or in series, are densely filled with minute bacilli. The invasion is recognizable under a low power in that the cytoplasm of the affected cell assumes a blue color when the section is stained in eosin-methylene blue (Fig. 1). High powers resolve this tint into fine, short, rod-like bodies (Figs. 2 to 6). The bacilli do not lie on the cell or in the ectoplasm but fill the cell body entirely. When the microscope is raised or lowered the cytoplasm appears filled in all optical sections.

The host cells are more or less altered as to size and condition of nucleus. Some cells are still normal in size and the nucleus is recog-

nizable as a vesicle with contained chromatin (Fig. 4). In others the nucleus is compact, pyknotic. The cell body becomes enlarged and it may measure up to 40 μ in diameter (Fig. 6). Vacuolation of the cytoplasm is not uncommon (Fig. 3). Fetal membranes at the end of the normal period of gestation which are macroscopically without pathological changes have been found uniformly free from such cell contents. The same is true of fetal membranes from cases of abortion associated with spirilla from which *Bacillus abortus* is not obtainable, either in cultures or through guinea pig inoculation.[1] In all cases in which the invasion of the epithelial cells was detected this feature was associated with positive cultures of *Bacillus abortus*, or the characteristic guinea pig disease after inoculation, or with both tests positive. Simple cover-slip preparations from the exudate or the necrotic villi were frequently sufficient to demonstrate the presence of infected epithelia. Besides the chorionic epithelium, the epithelial cells at the margin of the cotyledons and those of the outermost villi of the latter have been found invaded (Figs. 2 and 5). The bulk of the villi though undergoing profound changes are as a rule free from nests of bacteria.

That the cell localization described above is a regular occurrence wherever *B. abortus* is active may be gathered from the scanty literature by an interpretation of certain statements made, although none of the authors to be cited made an attempt to determine the precise source of the clumps of bacilli. Thus Bang[2] in 1897 describes the bacteria in films from the fetal membranes as either free or in dense clumps which appeared to have been formed inside cells. The following statement occurs in the British report:[3] "In many places the bacilli are collected into dense groups or colonies. Some of these groups look as if they were bounded by a cell membrane and give the impression of being contained inside tissue cells."[4] It is also stated[5] that: "It is an easy matter to identify the characteristic clumps of abortion bacilli in microscopic preparations made from the uterine exudate discharged immediately before or after abortion." In the Appendix[6] we read as

[1] Smith, T., *J. Exp. Med.*, 1918, xxviii, 701.

[2] Bang, B., *Z. Thiermed.*, 1897, i, 241.

[3] Great Britain Board of Agriculture and Fisheries, Report of the departmental committee on epizootic abortion, London, 1909, pt. 1.

[4] British report,[3] p. 6.

[5] British report,[3] p. 15.

[6] British report,[3] Appendix, p. 27.

follows: ". . . . so far as the membranes and swabs are concerned a positive diagnosis was based on the presence of the specific microbe in the typical clump form because these materials were always very impure and it is not possible to identify scattered abortion bacilli in a mixture of bacteria." Zwick and Zeller[7] refer to bacilli in large numbers in uterus exudate and state that cells may be filled with them. Fig. 3 illustrates very well what these writers probably refer to in most instances. In some cases, however, they may have seen leucocytes which at times are quite numerous and densely filled with bacilli. The latter are probably set free from the detached and disintegrated epithelial cells and taken in by leucocytes.

The mode of invasion of the epithelium by bacteria manifesting no true motility but only Brownian motion may be tentatively explained by assuming that the bacteria entering the uterochorionic space by way of the blood vessels in the uterine wall adhere to the ectoplasm and are rubbed into the substance of the cell by the pressure exerted by the uterine wall on the chorion. Once in the cytoplasm the bacteria find it a favorable medium for multiplication and a protection against phagocytosis. An alternative explanation would be to ascribe ameboid activities to the chorionic epithelium, for which theory at present no adequate proof exists.

It may be maintained that the cell parasitism is not an active disease process but rather secondary to it and operating only on cells partly or wholly devitalized. This interpretation has been kept in mind in the study of the various cases. When the disease is so far advanced that expulsion of the immature fetus occurs, the pathological material may fail to furnish an unequivocal answer to the above question. In several cases, however, in which a living calf at full term had fetal membranes in the early stages of infection, and in one slaughtered cow, the appearance of the invaded cells and the irregular distribution of the infection make it probable that they had been invaded while still in a normal condition (Figs. 2, 4, and 5).

The other types of epithelia, such as those of the uterine mucosa and of the amnion, have not been found invaded. The uterine epithelium resembles in its columnar appearance that of the chorion, but the cytoplasm forms a smooth, level, uninterrupted surface. The amniotic epithelium is of the flat, squamous type.

[7] Zwick and Zeller, *Arb. k. Gsndhtsamte.*, 1913, xliii, 1.

SUMMARY.

The significance of this invasion of the chorionic epithelium from the standpoint of pathogenesis cannot be properly evaluated until a more complete history of the successive localizations of *Bacillus abortus* has been obtained. It is safe to assume that this particular cell parasitism is but one of a series of localizations and centers of multiplication in the fetal membranes although evidence points to it as perhaps the earliest stage in which the organism gains by rapid, unchecked multiplication a considerable advantage over the host. The local destruction of an epithelial covering by an infectious agent when other miscellaneous infectious agents are absent may or may not be of much importance, for it would depend on the regenerative activity of the epithelium, the tendency to the gathering of injurious transudates, and the toxic substances associated with the bacilli.

It is probable that localizations also occur in the walls of the blood vessels of the chorion. Thus far only one case of this kind has been observed. The fusiform connective tissue cells of the adventitious coat of a blood vessel 0.8 mm. in diameter were completelylaced rep by clumps of minute bacilli. Since there is usually a slight perivascular cell infiltration in the diseased placenta this localization may be largely responsible for the circulatory disturbances which lead to death of the fetus. The case referred to may be but a greatly exaggerated illustration of the action of *Bacillus abortus* in the walls of the blood vessels where they are too few in number at any one time to be identified. It is known,[8,9] that in the guinea pig disease with pronounced lesions *Bacillus abortus* is demonstrated only with great difficulty because of its scarcity.

The more or less specific localization and multiplication of bacteria within cells not having phagocytic functions have thus far been demonstrated in leprosy, syphilis, and in a disease of mice recently described by Tyzzer[10] who found an active invasion of both liver cells and intestinal epithelium by a bacillus. In cells to which phagocytic powers have been ascribed the specific localization of certain bac-

[8] Smith, T., and Fabyan, M., *Centr. Bakteriol., 1te Abt., Orig.*, 1912, lxi, 549.

[9] Fabyan, M., *J. Med. Research*, 1912, xxvi, 441.

[10] Tyzzer, E. E., *J. Med. Research*, 1917–18, xxxvii, 307.

teria is well known. Thus tubercle bacilli occur within the endothelial cells of the tubercle. Leprosy bacilli have been found within a variety of cell groups. Mouse septicemia bacilli occur regularly within certain phagocytic cells of the blood.[11] Recently bacteria have been found attached to the cilia of the respiratory tract in pertussis by Mallory and Hornor,[12] in a form of guinea pig pneumonia by the writer.[13] Actual occupation of epithelial cells followed by active multiplication of the invaders and destruction of the cell has, however, been frequently demonstrated for the sporozoa. That it may occur more often among bacteria is highly probable. Rapidity of multiplication and cell destruction or invisibility or both may stand in the way of a satisfactory demonstration.

EXPLANATION OF PLATES.

Plate 20.

Fig. 1. Placenta of Heifer 203, which gave birth to a small but apparently healthy calf. Period of gestation unknown. The epithelium covering the chorion is enlarged, irregular in outline, the cytoplasm stained blue, and the nuclei are pyknotic. The cytoplasm of all is densely packed with bacilli not recognizable at this magnification. *B. abortus* was isolated from the placenta through guinea pigs. 10 days later the calf, having scoured somewhat, was killed and *B. abortus* was isolated from small foci of bronchopneumonia through guinea pigs. × 125.

Fig. 2. Placenta, Cow 146, aborted. A marginal villus of a cotyledon cut transversely and showing two epithelial cells *in situ* densely filled with bacilli. One cell is enlarged, projecting, the other very flat, cut transversely, and showing as a slender, nematode-like body. The nucleus of this latter infected cell is shown. × 1,000.

Fig. 3. Film made from placental exudate, Cow 298, aborted. Alkaline methylene blue. The epithelial cell is enlarged, vacuolated, and filled with bacilli. × 1,000.

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[11] Koch, R., Untersuchungen über die Aetiologie der Wundinfectionskrankheiten, Leipsic, 1878.

[12] Mallory, F. B., and Hornor, A. A., *J. Med. Research*, 1912–13, xxvii, 115.

[13] Smith, T., *J. Med. Research*, 1913–14, xxix, 291.

PLATE 21.

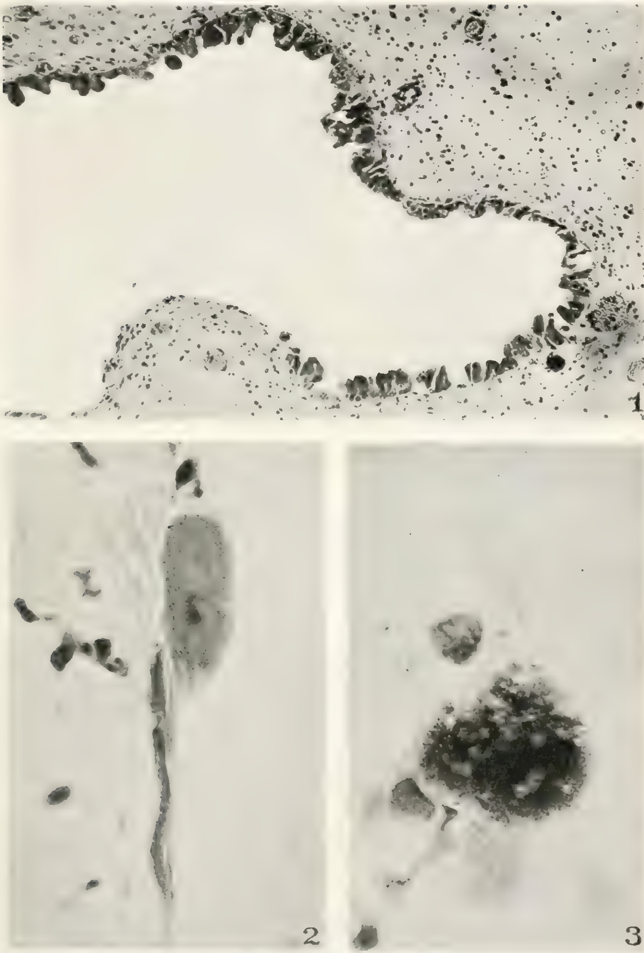
FIG. 4. Placenta, Cow 171. Fetus obtained *in utero* after slaughter. The chorionic epithelium is shown *in situ*. One densely infected cell in the center, others, right and left. The cell nuclei are no longer of normal appearance but the cells themselves are only slightly swollen. $\times$ 1,000.

FIG. 5. Placenta, Cow 91, aborted. A short blunt fetal villus from the margin of a cotyledon is shown with all of the covering epithelium filled with bacilli. $\times$ 1,000.

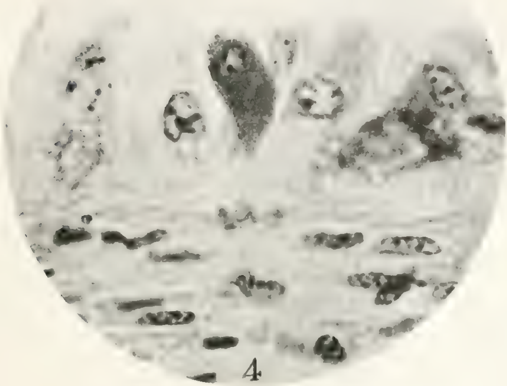
PLATE 22.

FIG. 6. Placenta, Cow 127, which gave birth to a full term calf. The epithelial cells of the chorion are slightly lifted away from their normal base. The cells are much larger than normal, the nuclei pyknotic. All are densely packed with bacilli. $\times$ 1,000.

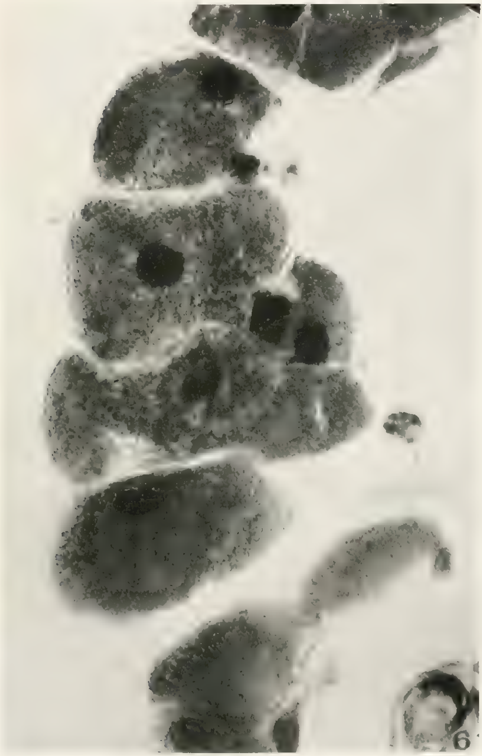
Guinea pigs inoculated with placental tissue of all the above cases, ground up and suspended in salt solution, became diseased and yielded pure cultures of *B. abortus*. In several cases cultures were obtained directly in spite of the soiled condition of placenta.



(Smith: Localization of *Bacillus abortus*.)



(Smith: Localization of *Bacillus abortus*.)



(Smith: Localization of *Bacillus abortus*.)

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